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ON THE PROTEOLYTIC ENZYMES OF ANIMAL TISSUES

III. THE PROTEOLYTIC ENZYMES OF BEEF SPLEEN, BEEF KIDNEY, AND SWINE KIDNEY. CLASSIFICATION OF THE CATHEPSINS

By JOSEPH S. FRUTON, GEORGE W. IRVING, JR., AND
MAX BERGMANN

(From the Laboratories of The Rockefeller Institute for Medical Research)

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Aqueous extracts of beef spleen have been shown to contain at least three proteolytic enzymes of widely different specificity (1). Beef spleen Cathepsin I hydrolyzes carbobenzoxy-*l*-glutamyl-*l*-tyrosine and does not require the addition of an activator such as cysteine. Beef spleen Cathepsin II hydrolyzes benzoyl-*l*-arginineamide when an activator such as cysteine is added. Beef spleen Cathepsin III hydrolyzes *l*-leucineamide in the presence of activators such as cysteine or ascorbic acid. In the present communication, the identification of a fourth proteolytic component (Cathepsin IV) of beef spleen cathepsin is reported. Furthermore, counterparts of these four enzymes have also been found in beef kidney and swine kidney.

Identification of a New Proteolytic Enzyme in Beef Spleen Cathepsin

An indication for the presence of a fourth proteolytic component in beef spleen cathepsin was obtained by a comparative study of the action of a cysteine-activated spleen extract upon benzoyl-*l*-arginineamide, benzoyl-*l*-lysineamide, carbobenzoxyglycyl-*l*-phenylalanine, and carbobenzoxyglycyl-*l*-tyrosine. It was previously reported (2) that Cathepsin II is unstable at pH values more acid than 4. It will be noted from the data in Table I that the activity of beef spleen cathepsin toward carbobenzoxyglycyl-phenylalanine is more resistant to acidity than is the activity of

Cathepsin II. It must be concluded that carbobenzoxylglycyl-*l*-phenylalanine is hydrolyzed by a cysteine-activatable enzyme other than Cathepsin II. This component is the Cathepsin IV mentioned above. The data in Table I indicate also that at pH 3.5 and 3.9 the activities of spleen cathepsin toward benzoyl-arginineamide and benzoyllysineamide decreased in a parallel manner. This indicates that benzoyllysineamide is split by the same enzyme as is benzoylarginineamide; *i.e.*, Cathepsin II.

TABLE I

Effect of Increased Acidity on Activity of Beef Spleen Cathepsin

Samples of a solution of beef spleen cathepsin (1.40 mg. of protein N per cc. of enzyme solution) were adjusted to pH 3.9 or 3.5 with *N* acetic acid and kept at these pH values for 1 hour at 40°. The pH of the enzyme solutions was then brought to 5.0 with *N* NaOH. In a control experiment a mixture of *N* acetic acid and *N* NaOH was made up in the proportions applied above and then added to the enzyme. The control enzyme solution was also kept at 40° for 1 hour. The three above enzyme solutions were tested for enzymatic activity in the presence of cysteine (0.01 mm per cc. of test solution). The pH of the test solution was 4.8; the temperature was 40°.

Substrate	$C^* \times 10^3$		
	Control	Treated enzyme	
		pH 3.9	pH 3.5
Benzoyl- <i>l</i> -arginineamide (4)	8.3	5.2	2.8
Benzoyl- <i>l</i> -lysineamide (5)	4.0	2.4	1.3
Carbobenzoxylglycyl- <i>l</i> -phenylalanine (6)	4.2	4.1	3.5

$$* C = \frac{K \text{ (first order)}}{\text{mg. protein N per cc. test solution}} \quad (3).$$

A sample of beef spleen cathepsin was dialyzed against distilled water, and its activity upon the previously mentioned substrates was tested before and after dialysis. The data in Table II show that on dialysis the proteolytic coefficient toward benzoylarginineamide rises slightly,¹ while the proteolytic coefficients toward carbobenzoxylglycylphenylalanine and carbobenzoxylglycyltyrosine drop markedly. This decrease is of the same magnitude for both substrates. It may therefore be concluded that the hydrolysis of carbobenzoxylglycylphenylalanine and carbobenzoxylglycyltyrosine is due to the same enzymatic component of spleen cathepsin; *i.e.*, Cathepsin IV.

¹ This increase is due to the loss of protein other than Cathepsin II.

It had previously been found (1) that carbobenzoxy-*l*-glutamyl-*l*-phenylalanine is hydrolyzed by two different enzymatic components of beef spleen cathepsin, one of which (Cathepsin I) is effective in the absence of an added activator, while the other is activated by cysteine. It has now been found that the activity of this second, cysteine-activatable enzyme was not diminished when the beef spleen extract was kept at pH 3.9 and 40° for 1 hour, but that this enzyme is lost when the spleen extract is dialyzed against distilled water for 48 hours. This behavior is similar to that previously found for Cathepsin IV, and it may be assumed that the cysteine-activatable beef spleen component that acts on carbobenzoxyglutamylphenylalanine is identical with the component

TABLE II

Loss of Cathepsin IV Activity on Dialysis of Beef Spleen Cathepsin

A solution of beef spleen was dialyzed against 1 per cent sodium chloride. The resulting Solution A was then dialyzed against distilled water for 48 hours at 4° to give an enzyme Solution B. Solutions A and B were tested for proteolytic activity with cysteine as activator (0.01 mm per cc. of test solution). Temperature, 40°; pH 4.8 to 5.1.

Substrate	$C \times 10^3$		Cathepsin component
	Enzyme Solution A	Enzyme Solution B	
Benzoyl- <i>l</i> -arginineamide	8.0	9.1	II
Carbobenzoxyglycyl- <i>l</i> -phenylalanine	3.7	1.4	IV
Carbobenzoxyglycyl- <i>l</i> -tyrosine (7)	2.3	0.9	"

that hydrolyzes carbobenzoxyglycylphenylalanine; namely, Cathepsin IV. This tentative conclusion cannot, at present, be subjected to a decisive test by comparing, under various experimental conditions, the reaction rates of the cysteine-activated catheptic hydrolyses of carbobenzoxyglycylphenylalanine and carbobenzoxyglutamylphenylalanine. Since the latter substrate is hydrolyzed simultaneously by two spleen components, rate constants for its hydrolysis cannot be calculated.

Cathepsins of Beef Kidney and Swine Kidney

Cathepsin I—Similarly to beef spleen, extracts of beef kidney and swine kidney contain enzymes (beef kidney Cathepsin I and swine kidney Cathepsin I) that hydrolyze carbobenzoxy-*l*-glutamyl-*l*-tyrosine in the absence of added activators. These

enzymes are rapidly inactivated at 50°. This thermolability was utilized to show that Cathepsin I in beef kidney and swine kidney hydrolyzes not only carbobenzoxyglutamyltyrosine but also the compound carbobenzoxy-*l*-glutamyl-*l*-phenylalanine. It will be noted in Table III that after the enzyme solutions were heated at 50° for 15 minutes the activity toward the two substrates decreased to the same degree.

Cathepsin II—The substrate for cysteine-activated beef spleen Cathepsin II, benzoyl-*l*-arginineamide, is also hydrolyzed by beef kidney and swine kidney extracts after cysteine has been added as the activator. The enzymes responsible for these hydrolyses are

TABLE III
Heat Inactivation of Cathepsin I

Solution A of beef kidney cathepsin (1.55 mg. of protein N per cc. of enzyme solution) was heated at 50° for 15 minutes and then was chilled in ice water to give a beef kidney cathepsin solution (B). A Solution A of swine kidney cathepsin (1.10 mg. of protein N per cc. of enzyme solution) was also treated as above to yield a swine kidney cathepsin solution (B). 0.5 cc. of the enzyme Solutions A and B was employed for the hydrolytic experiments. No cysteine was added. Temperature, 25°; pH 5.3 to 5.5.

Enzyme solution	Carbobenzoxy- <i>l</i> -glutamyl- <i>l</i> -tyrosine (8)		Carbobenzoxy- <i>l</i> -glutamyl- <i>l</i> -phenylalanine	
	$K \times 10^4$	$\frac{K(B)}{K(A)}$	$K \times 10^4$	$\frac{K(B)}{K(A)}$
Beef kidney, A.	6.2		2.6	
“ “ B.	3.0	0.48	1.2	0.46
Swine “ A.	7.0		3.5	
“ “ B.	5.3	0.76	2.6	0.74

designated beef kidney Cathepsin II and swine kidney Cathepsin II. The first order velocity constants for the hydrolysis effected by these enzymes are proportional to the enzyme concentration within the limits employed (Table IV). The value of the proteolytic coefficient for benzoylarginineamide (C_{BAA}) for beef spleen extract was previously found to be about 0.008, corresponding to 4 enzyme units per mg. of protein nitrogen, when an enzyme unit is defined according to a previous paper (3). On the same basis, the beef kidney extract used in the experiments reported in Table IV contained 3.6 units per mg. of protein nitrogen, while the swine kidney extract contained 12 units per mg. of protein nitrogen.

Beef kidney Cathepsin II and swine kidney Cathepsin II were also found to hydrolyze benzoyl-*l*-lysineamide. The experimental data will be presented in a succeeding section of this paper.

Cathepsin III—Both beef kidney and swine kidney extracts contain an enzyme (Cathepsin III) that hydrolyzes *l*-leucineamide and *l*-leucylglycine at pH 5 when ascorbic acid or cysteine has been

TABLE IV

*Hydrolysis of Benzoyl-*l*-arginineamide by Cathepsin II of Beef and Swine Kidney*

0.01 mm of cysteine per cc. of test solution. Temperature, 40°; pH 4.7.

Source of enzyme	Protein N per cc. test solution	$K \times 10^4$	$C \times 10^4$
	mg.		
Beef kidney	0.13	9	6.9
	0.20	14	7.0
	0.33	24	7.3
Swine kidney	0.05	12	24
	0.10	26	26
	0.21	51	24

TABLE V

Comparison of Cathepsin III Activity in Beef Spleen, Beef Kidney, and Swine Kidney

Temperature, 40°; pH 5.0 to 5.2.

Substrate	Activator, 0.01 mm per cc. test solution	Beef spleen cathepsin*		Beef kidney cathepsin†		Swine kidney cathepsin†	
		$K \times 10^4$	$C \times 10^3$	$K \times 10^4$	$C \times 10^3$	$K \times 10^4$	$C \times 10^3$
<i>l</i> -Leucineamide	Cysteine	33	9.2	3.0	1.0	11	3.7
	Ascorbic acid	7	1.9	0.6	0.2	2.2	0.7
<i>l</i> -Leucylglycine	Cysteine	16	4.4	1.2	0.4	5	1.7

* 0.36 mg. of protein N per cc. of test solution.

† 0.30 mg. of protein N per cc. of test solution.

added as activator (Table V). It will be noted that the Cathepsin III activity per mg. of protein nitrogen is much smaller in beef and swine kidney than in beef spleen.

Cathepsin IV—Extracts of beef kidney and swine kidney have been found to contain cysteine-activatable enzymes (beef kidney Cathepsin IV and swine kidney Cathepsin IV) that hydrolyze

carbobenzoxyglycylphenylalanine. Swine kidney extracts show a particularly high Cathepsin IV activity. It may be calculated from the data in Table VI that beef spleen extracts contain 1.5 Cathepsin IV units per mg. of protein nitrogen, beef kidney 3 Cathepsin IV units, and swine kidney 16.5 Cathepsin IV units.

TABLE VI

Hydrolysis of Carbobenzoxyglycyl-l-phenylalanine by Beef Spleen, Beef Kidney, and Swine Kidney

Cysteine, 0.01 mm per cc. of test solution. Temperature, 40°; pH 4.9.

Source of enzyme	Protein N per cc. test solution	$K \times 10^4$	$C \times 10^4$
	mg.		
Beef spleen	0.14	4	2.9
	0.28	8	2.9
	0.56	15	2.7
" kidney	0.21	12	5.7
	0.28	16	5.7
	0.42	25	6.0
Swine "	0.05	16	32
	0.10	34	34
	0.21	71	34

TABLE VII

Effect of Cysteine on Hydrolysis of Carbobenzoxy-l-glutamyl-l-tyrosine
Temperature, 25°; pH 5.3 to 5.5.

Source of enzyme	Protein N per cc. test solution	Hydrolysis in 2 hrs.	
		No cysteine	Cysteine*
	mg.	per cent	per cent
Beef spleen.	0.29	22	24
" kidney	0.31	15	37
Swine "	0.18	10	36

* 0.01 mm per cc. of test solution.

In an earlier section of this paper evidence was presented to indicate that Cathepsin IV, when activated, hydrolyzes the typical substrates of Cathepsin I (carbobenzoxy-l-glutamyl-l-phenylalanine and carbobenzoxy-l-glutamyl-l-tyrosine): This explains why tissues that are relatively rich in Cathepsin IV show a marked

increase in the hydrolysis of carbobenzoxyglutamyltyrosine on addition of cysteine (Table VII). Beef spleen, which is relatively poor in Cathepsin IV, shows no appreciable increase in the hydrolysis of carbobenzoxyglutamyltyrosine when cysteine is added.

Classification of Cathepsins in Revised System of Proteolytic Enzymes

In the past, proteolytic enzymes were classified as dipeptidases, polypeptidases, and proteinases (9). Consequently, the catheptic enzymes, as classified according to this scheme, included one dipeptidase, one aminopolypeptidase, one carboxypolypeptidase, and one proteinase (10). However, during recent years information has been gathered that requires revision of the above classification. In particular, the specificity and mechanism of the action of proteinases has been studied further (11). Moreover, it has been observed repeatedly that aminopeptidases and carboxypeptidases may also split dipeptides, and consequently the existence of a separate group of dipeptidases may be questioned (6, 12-14).

The revised classification proposed in Table VIII is based on the nature and position of the chemical groups in the peptide chain of the substrate that are required for the action of various proteolytic enzymes. However, the sensitivity of a substrate to a given proteolytic enzyme is determined not only by the groups in the peptide chain of the substrate but also by the nature of the constituent amino acids. Consequently, each of the classes in Table VIII may be subdivided further on the basis of the amino acid residues in the substrates that are essential for the action of specific enzymes.

It will be noted that pepsin and trypsin belong to the group of carbonylproteinases, while chymotrypsin is an imidoproteinase. The Cathepsins I of beef spleen, beef kidney, and swine kidney fall into the group of carbonylproteinases. This conclusion is based on the fact that glutamyltyrosine is not split by these enzymes and no substrate has been found for them that does not have a peptide linkage adjacent to the carbonyl side of the sensitive peptide bond. Cathepsin II is also a carbonylproteinase. Cathepsin III splits *l*-leucineamide besides *l*-leucylglycine and therefore must be an aminopeptidase. Cathepsin IV appears to be a carboxypeptidase, since carbobenzoxyglycylphenylalanineamide is not split appreciably by a cysteine-activated swine kidney extract that is rich

TABLE VIII
Revised Classification of Proteolytic Enzymes

Class	Enzyme	Requisite groups in peptide chain
Peptidases (exo-peptidases (15))		
Amino-pepti- dases	Intestinal amino-pepti- tidase Cathepsin III	$\begin{array}{c} \text{R} \\ \\ \text{NH}_2\text{---CH---CO---NH}\cdots \\ \Downarrow \end{array}$
Carboxypepti- dases	Pancreatic carboxy- peptidase Cathepsin IV	$\begin{array}{c} \text{R} \\ \\ \text{NH}_2\text{---CH---COOH} + \text{NH}_2\cdots \\ \text{---CO---NH---CH---COOH} \\ \\ \Downarrow \\ \text{R} \\ \\ \text{---COOH} + \text{NH}_2\text{---CH---COOH} \end{array}$
Proteinases (endo-peptidases (15))		
Carbonylpro- teinases	(a) Pepsin Cathepsin I	$\begin{array}{c} \text{R} \\ \\ \cdots\text{CO---NH---CH---CO---NH}\cdots \\ \Downarrow \end{array}$
	(b) Trypsin Papain* Cathepsin II	$\begin{array}{c} \text{R} \\ \\ \cdots\text{CO---NH---CH---COOH} + \text{NH}_2\cdots \end{array}$
Imidoprotein- ases	Chymotryp- sin†	$\begin{array}{c} \text{R} \\ \\ \cdots\text{CO---NH---CH---CO---NH}\cdots \\ \Downarrow \\ \text{R} \\ \\ \cdots\text{COOH} + \text{NH}_2\text{---CH---CO---NH}\cdots \end{array}$

* The component of papain that hydrolyzes benzoyl-*l*-arginineamide.

† Chymotrypsin is designated an imidoproteinase because it hydrolyzes *l*-tyrosylglycineamide at the peptide linkage joining the tyrosyl and glycyl residues (unpublished experiments) and also hydrolyzes carbobenzoxy-tyrosylglycineamide (7).

in Cathepsin IV and highly active toward carbobenzoxyglycyl-phenylalanine.

Additional support for this classification has been obtained by comparing the action of several enzymes upon two test substrates in a quantitative manner. Thus, the Cathepsins II of beef spleen, beef kidney, and swine kidney are compared with respect to their action on benzoylarginineamide and benzoyllysineamide (Table IX). The proteolytic quotient C_{BAA}/C_{BLA} was found to be essentially the same (2.2 to 2.5). New determinations of the reaction velocity constants for the hydrolysis of the above substrates by

TABLE IX
Hydrolysis of Benzoyl-L-arginineamide and Benzoyl-L-lysineamide by Several Proteolytic Enzymes

In all cases, except that of trypsin, cysteine was present in a concentration of 0.01 mm per cc. of test solution and the temperature was 40°. In the experiments with trypsin, the temperature was 25°.

Enzyme	pH	$C \times 10^4$		$\frac{C_{BAA}}{C_{BLA}}$
		Benzoyl-arginineamide	Benzoyllysineamide	
Beef spleen Cathepsin II.	4.7	8.3	3.8	2.2
“ kidney “ “	4.7	8.7	3.7	2.3
Swine “ “ “	4.7	27	11	2.5
Trypsin.	7.4	42	20	2.1
Papain.	5.0	167	78	2.1

crystalline beef trypsin (*cf.* also (5)) gave a proteolytic quotient C_{BAA}/C_{BLA} of 2.1. Furthermore, with cysteine-papain a proteolytic quotient of C_{BAA}/C_{BLA} of 2.1² was obtained.

The similarity of the proteolytic quotients C_{BAA}/C_{BLA} for the five enzymes is the more striking since the enzymes compared differ in their pH optimum and activation behavior. In our opinion, this similarity exists because the five enzymes all act upon their

² It was found that the proteolytic activity toward each substrate dropped to 30 per cent of the original value after treatment of a papain solution at pH 2 for 17 hours at 21°. The fact that the quotient C_{BAA}/C_{BLA} was unchanged indicates that the two substrates are split by the same enzymatic component of papain.

substrates by a similar reaction mechanism; they all are carboxylproteinases.

The hydrolysis of the substrates benzoylglycyl-*l*-arginineamide and benzoylglycyl-*l*-lysineamide by crystalline trypsin has already

TABLE X

Comparison of Cathepsins IV with Crystalline Carboxypeptidase

In all cases, except that of carboxypeptidase, cysteine was present in a concentration of 0.01 mm per cc. of test solution and the temperature was 40°. In the experiments with carboxypeptidase, the temperature was 25°.

Enzyme	pH	$C \times 10^3$		$\frac{C_{CGP}}{C_{CGT}}$
		Carbo- benzoxy- glycyl- <i>l</i> - phenylala- nine	Carbo- benzoxy- glycyl- <i>l</i> - tyrosine	
Beef spleen Cathepsin IV	5.0	2.5	1.5	1.7
“ kidney “ “	5.1	6.3	4.0	1.6
Swine “ “ “	5.0	34	19	1.8
Carboxypeptidase*	7.7	6570	3620	1.8

* This preparation was kept at 0° for over 1 year and thus had lost some activity.

TABLE XI

Comparison of Cathepsin I Activity in Beef Spleen, Beef Kidney, and Swine Kidney

No cysteine was added. Temperature, 25°; pH 5.3 to 5.5.

Source of enzyme	Protein N per cc. test solution	Carbobenzoxy- <i>l</i> -glu- tamyl- <i>l</i> -phenylalanine		Carbobenzoxy- <i>l</i> - glutamyl- <i>l</i> -tyrosine		$\frac{C_{CGP}}{C_{CGT}}$
		$K \times 10^4$	$C \times 10^3$	$K \times 10^4$	$C \times 10^3$	
	mg.					
Beef spleen	0.45	7.2	1.6	14.7	3.3	0.48
“ kidney . .	0.31	2.6	0.84	6.2	2.0	0.42
Swine “ . .	0.22	3.5	1.6	7.0	3.2	0.50

been studied (16) and the quotient C_{BGAA}/C_{BGLA} was found to be 1.8. This value is close to those given above for the quotient C_{BAA}/C_{BLA} .

In Table X the Cathepsins IV of beef spleen, beef kidney, and swine kidney as well as crystalline carboxypeptidase from beef

pancreas are compared with respect to their action on carbobenzyglycyl-*l*-phenylalanine and carbobenzyglycyl-*l*-tyrosine. It will be noted that the proteolytic quotient $C_{\text{CGP}}/C_{\text{CGT}}$ was found to be 1.6 to 1.8. Here again enzymes are compared which differ with respect to pH optima and activation behavior but which belong to the same group of carboxypeptidases.

The proteolytic quotients $C_{\text{CGP}}/C_{\text{CGT}}$ for the hydrolysis of carbobenzy-*l*-glutamyl-*l*-phenylalanine and carbobenzy-*l*-glutamyl-*l*-tyrosine by the Cathepsins I of beef spleen, beef kidney, and swine kidney were found to be 0.42, 0.48, and 0.50, respectively (Table XI), thus indicating that the phenylalanine-containing substrate is hydrolyzed at a rate one-half of that of the tyrosine-containing substrate. It will be recalled that in the case of Cathepsin IV the phenylalanine-containing substrate was hydrolyzed at nearly twice the rate of the hydrolysis of the tyrosine-containing substrate. This difference in the quotients for Cathepsin I and Cathepsin IV indicates that these two enzymes have different mechanisms of action, and serves as added support for the classification of Cathepsin I and Cathepsin IV in separate classes (Table VIII).

The authors wish to express their thanks to Mr. Maurice Rapport for valuable assistance in this investigation.

EXPERIMENTAL

The beef spleen cathepsin solutions were prepared as described in a previous paper (2). The same procedure was employed for the preparation of beef kidney cathepsin and swine kidney cathepsin. Crystalline trypsin was prepared according to the directions of Kunitz and Northrop (17). Crystalline carboxypeptidase was prepared by the method of Anson (18). The papain preparation was obtained as described in a previous paper (19).

The course of enzymatic hydrolysis was followed by means of amino nitrogen determinations and the microtitration method of Grassmann and Heyde. The substrate concentration was 0.05 mm per cc. of the test solution in all cases. The pH was adjusted by means of citrate buffers (near pH 5) and by means of phosphate buffers (near pH 7).

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THE SPECIFIC ROTATION OF *L*-TYROSINE

BY WILLIAM H. STEIN, STANFORD MOORE, AND MAX BERGMANN

(From the Laboratories of The Rockefeller Institute for Medical Research)

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The values for the specific rotation of *L*-tyrosine obtained in different laboratories show considerable variation. For example, in 4% hydrochloric acid, Schulze and Winterstein^{1,2} reported $[\alpha]_D^{16} = -16.1^\circ$ and -16.2° , Fischer³ $[\alpha]_D^{20} = -13.2^\circ$, Bergmann and Zervas⁴ $[\alpha]_D^{22} = -12.44^\circ$, and Dudley⁵ $[\alpha]_D = -11.6^\circ$.

We have had occasion to prepare tyrosine by the following procedures: (a) digestion of casein by pancreatin; (b) hydrolysis of silk fibroin with concentrated hydrochloric acid; (c) recrystallization of a commercial sample of *L*-tyrosine employing hydrochloric acid and ammonium acetate; (d) repeated recrystallization of *L*-tyrosine 4-nitrotoluene-2-sulfonate⁶ and regeneration of the amino acid; (e) repeated recrystallization of *L*-tyrosine 3-carboxy-4-hydroxyazobenzene sulfonate⁷ and regeneration of the amino acid; (f) resolution of synthetic benzoyl-*DL*-tyrosine⁸ and subsequent hydrolysis in the manner described by Fischer.³ The six samples possessed the same specific rotation, $[\alpha]_D^{26} = -10.3^\circ \pm 0.2^\circ$ ($C = 5.00$; 4% HCl). This indicates that the observed rotation is that of pure *L*-tyrosine.

The rotation, -10.3° , was determined at the relatively high temperature of 26° . Consequently the specific rotation of our samples was also determined at lower temperatures. The results are given in the accompanying table. It will be noted that in 4% hydrochloric acid the rotation varies considerably with temperature, and that this variation is approximately linear over the room temperature range. Minor changes in hydrochloric acid or tyrosine concentration, however, are not significant sources of variation. The specific rotation in 20% hydrochloric acid, for which Fischer³ reported $[\alpha]_D^{20} = -8.64^\circ$,

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- (7) The amino acid salts of carboxyhydroxyazobenzenesulfonic acid will be described in a forthcoming paper.
- (8) The benzoyl-*L*-tyrosine obtained as intermediate in this procedure had the same specific rotation as reported by Fischer.

is similarly sensitive to temperature. The value of the specific rotation of tyrosine is a function, therefore, not only of the purity of the sample, but also of the adequacy of the temperature control.

In connection with these findings, it may be noted that Fischer³ reported $[\alpha]_D^{20} - 12.56^\circ$ for a sample of tyrosine prepared from casein after hydrolysis with 20% hydrochloric acid. This value was slightly lower than the specific

TABLE I

Tyrosine concn., %	HCl concn., %	Temp., °C.	$[\alpha]_D$
5	4	26 ± 0.3	10.3 ± 0.2
5	4	$20 \pm .3$	$11.8 \pm .2$
5	4	$16 \pm .3$	$13.0 \pm .2$
4	20	$26 \pm .3$	$7.0 \pm .2$
4	20	$20 \pm .3$	$8.5 \pm .2$
4	20	$16 \pm .3$	$9.6 \pm .2$

rotation, $[\alpha]_D^{20} - 13.2^\circ$, observed by Fischer for his synthetic *l*-tyrosine. Fischer attributed this difference to the presence of inactive tyrosine in the sample obtained from casein. In view of the sensitivity of the specific rotation of tyrosine to changes in temperature, however, the question of the racemization by strong acids warrants further investigation.

The assistance of Miss J. E. Tietzman is gratefully acknowledged by the authors.

AROMATIC SULFONIC ACIDS AS REAGENTS FOR AMINO ACIDS

THE PREPARATION OF *l*-SERINE, *l*-ALANINE, *l*-PHENYLALANINE, AND *l*-LEUCINE FROM PROTEIN HYDROLYSATES

BY WILLIAM H. STEIN, STANFORD MOORE, GUIDO STAMM,*
CHI-YUAN CHOU,† AND MAX BERGMANN

(From the Laboratories of The Rockefeller Institute for Medical Research)

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A number of sulfonic acids of the benzene and naphthalene series have recently been recommended as reagents for amino acids (1). The experiments described in the present communication extend the earlier work to sulfonic acids derived from diphenylamine, anthraquinone, and azobenzene and also illustrate how a number of these reagents may be employed to advantage for the preparation of amino acids from protein hydrolysates.

The structure of a number of the sulfonic acids investigated, together with the solubility products of their amino acid salts, is given in Table I. In general the solubilities listed in Table I are lower than those of the amino acid salts of the benzene- and naphthalenesulfonic acids previously reported. Several of the reagents warrant special mention. The *l*-isoleucine salt of 4-nitro-4'-methyldiphenylamine-2-sulfonic acid (Reagent 7) is considerably less soluble than are the corresponding *l*-leucine and *l*-phenylalanine salts. Its application to the isolation and determination of isoleucine, therefore, may merit investigation. Five of the azobenzenesulfonic acids (Reagents 1, 2, 4, 5, and 6) and the three anthraquinonesulfonic acids (Reagents 10, 11, and 12) all form sparingly soluble salts with most of the amino acids tested. Remarkably low in solubility are the arginine and histidine salts of 3-carboxy-4-hydroxyazobenzene-4'-sulfonic acid (Reagent 4) and 2-hydroxy-5-methylazobenzene-3'-sulfonic acid (Reagent 6) and the glycine, arginine, histidine, and lysine salts of 5-nitroanthraquinone-1-sulfonic acid. Among this group of reagents *p*-hydroxyazobenzene-*p*'-sulfonic acid (Reagent 2) is also noteworthy because it is capable of precipitating *l*-serine. This reagent has been utilized for the isolation of *l*-serine from silk fibroin (2). It is interesting that the isomeric *p*-hydroxyazobenzene-*m*'-sulfonic acid (Reagent 3) behaves entirely differently. In this case the *l*-phenylalanine salt is much less soluble than are any of the

* Fellow of the Institute of International Education.

† Fellow of the Peiping Union Medical College.

other amino acid salts. The utility of this substance is impaired, however, by the insolubility of the free sulfonic acid itself. Finally, it should be noted that many of the aforementioned azo compounds, even in strongly acidic solutions, form salts with a number of organic bases such as

TABLE I—Solubility Products of Amin

The solubilities were determined at 0°. It should be emphasized that all the in the experimental section. Where no value is given, no precipitation was obtained.

Amino acid	(1) Azobenzene- <i>p</i> -*	(2) <i>p</i> -Hydroxy- azobenzene- <i>p'</i> -†	(3) <i>p</i> -Hydroxy- azobenzene- <i>m'</i> -‡	(4) 3-Carboxy-4- hydroxyazo- benzene-4'-	(5) 3-Carboxy-4- hydroxyazo- benzene-3'-
<i>L</i> -Alanine	4×10^{-4}	2×10^{-3}			
Ammonia	4×10^{-2}	4×10^{-5}	3×10^{-4}	3×10^{-3}	3×10^{-4}
<i>L</i> -Arginine**	2×10^{-8}	2×10^{-8}		1×10^{-9}	5×10^{-9}
<i>L</i> -Aspartic acid					
<i>L</i> -Cysteine.	2×10^{-5}	8×10^{-6}		4×10^{-6}	1×10^{-5}
<i>L</i> -Cystine**	1×10^{-6}	8×10^{-8}		2×10^{-9}	3×10^{-7}
<i>L</i> -Glutamic acid		4×10^{-3}			
Glycine	2×10^{-4}	1×10^{-3}		3×10^{-2}	5×10^{-4}
<i>L</i> -Histidine**	3×10^{-8}	2×10^{-9}		7×10^{-10}	2×10^{-9}
<i>L</i> -Hydroxyproline					2×10^{-3}
<i>L</i> -Isoleucine	4×10^{-4}			2×10^{-4}	6×10^{-4}
<i>L</i> -Leucine	8×10^{-5}	3×10^{-4}		1×10^{-5}	1×10^{-4}
<i>L</i> -Lysine**	1×10^{-7}	3×10^{-8}		1×10^{-8}	2×10^{-8}
<i>dl</i> -Methionine	6×10^{-5}	2×10^{-4}		4×10^{-5}	1×10^{-5}
<i>L</i> -Phenylalanine	1×10^{-5}	6×10^{-6}	4×10^{-5}	1×10^{-5}	4×10^{-5}
<i>L</i> -Proline		3×10^{-4}			
<i>L</i> -Serine		2×10^{-4}			
<i>dl</i> -Threonine					
<i>L</i> -Tyrosine.		1×10^{-4}		8×10^{-6}	2×10^{-5}
<i>L</i> -Valine	1×10^{-4}			1×10^{-3}	

* 0.3 cc. of methyl cellosolve added to each sample to dissolve the free sulfonic acid.

† Sulfonic acid dissolved in 40 per cent methyl cellosolve.

‡ Sulfonic acid dissolved in 50 per cent methyl cellosolve, and 1 cc. of water and

§ 0.1 cc. of methyl cellosolve added to each sample to dissolve the free sulfonic

|| 0.2 cc. of methyl cellosolve and 1 cc. of water added to each sample to dissolve

¶ Sulfonic acid dissolved in *N* HCl; solubilities determined in *N* HCl.

** The values given for these amino acids represent the solubility products of the

piperidine, aniline, pyridine, hydroxylamine, β -aminophenol, ethanolamine, creatine, and urea.

Picrylsulfonic acid (Reagent 13), like picric acid, forms sparingly soluble salts with ammonia, the three basic amino acids, and with glycine and proline. This reagent, which in contrast to picric acid may be employed

in strongly acid solution, gives promise of being useful for the determination of proline.

The following sulfonic acids have also been investigated: 2,6-dinitro-2'-methyldiphenylamine-4-, 2,6-dinitro-3'-methyldiphenylamine-4-, 4,6-

o Acid Salts of Aromatic Sulfonic Acids

solubility products are approximate values only, estimated in the manner described at concentrations corresponding to a solubility product of 4×10^{-3} .

Solubility products of sulfonic acid salts

(6) 2-Hydroxy- 5-methylazo- benzene-3'-4	(7) 4-Nitro-4'- methyldiph- enylamine-2-	(8) 2-Nitro-4'- methyldiph- enylamine-4-	(9) 4-Nitro- diphenyl- amine-2-	(10) Anthraqui- none- α -	(11) Anthraqui- none- β -	(12) 5-Nitro- anthraqui- none-1-	(13) Picryl-¶
3×10^{-3}	4×10^{-2}	2×10^{-3}	4×10^{-4}	3×10^{-3}	2×10^{-3}	6×10^{-4}	
4×10^{-10}	Oil	4×10^{-9}	3×10^{-7}	6×10^{-6}	3×10^{-3}	1×10^{-4}	4×10^{-4}
				2×10^{-8}	3×10^{-9}	2×10^{-9}	8×10^{-6}
2×10^{-6}		2×10^{-3}		6×10^{-4}		4×10^{-5}	
6×10^{-8}	Oil		Oil	8×10^{-7}	3×10^{-8}	8×10^{-9}	
1×10^{-2}							
3×10^{-5}			5×10^{-4}	2×10^{-4}	1×10^{-2}	4×10^{-6}	3×10^{-3}
5×10^{-9}	2×10^{-8}	2×10^{-8}	3×10^{-7}	3×10^{-9}	3×10^{-8}	8×10^{-9}	2×10^{-5}
1×10^{-2}						3×10^{-3}	
1×10^{-3}	8×10^{-6}	1×10^{-4}		3×10^{-4}	2×10^{-4}	3×10^{-3}	
8×10^{-6}	2×10^{-4}	8×10^{-5}	5×10^{-4}	5×10^{-4}	1×10^{-4}	2×10^{-5}	
1×10^{-7}	Oil	4×10^{-7}	6×10^{-9}	6×10^{-8}	1×10^{-6}	3×10^{-9}	3×10^{-4}
7×10^{-6}	7×10^{-6}	4×10^{-5}		1×10^{-4}	3×10^{-3}	2×10^{-4}	
1×10^{-6}	5×10^{-4}	2×10^{-4}	3×10^{-5}	1×10^{-3}	3×10^{-5}	6×10^{-5}	
8×10^{-4}	3×10^{-5}					3×10^{-4}	5×10^{-4}
				2×10^{-3}		4×10^{-3}	
						6×10^{-4}	
					2×10^{-3}		
4×10^{-3}	1×10^{-5}	8×10^{-5}		1×10^{-3}	4×10^{-3}	1×10^{-3}	

0.6 cc. of methyl cellosolve added to each sample to dissolve the reagent.
acid.

the free sulfonic acid.

ternary salts (see foot-note to Table I (1)).

dinitrodiphenylamine-2-, 4,6-dinitro-2'-methyldiphenylamine-2-,
2-nitro-3'-methyldiphenylamine-4-, 2-nitro-4'-ethoxydiphenylamine-4-,
4-nitrodiphenylamine-2-, 2-benzylamino-5-nitrobenzene-, and 4-nitro-4'-
methyldiphenylamine-2-. Their amino acid salts possess, in general,
solubilities similar to those of the reagents given in Table I.

The utilization of sulfonic acids for the preparation of amino acids may be exemplified by the procedures for the isolation of *l*-phenylalanine and *l*-leucine from a hydrolysate of hemoglobin, and of *l*-serine and *l*-alanine from a hydrolysate of silk fibroin.

In the course of the preparation of lysine (3) and histidine from hemoglobin an insoluble amino acid fraction is obtained. From this by-product we have prepared, per kilo of commercial hemoglobin, about 48 gm. of *l*-leucine (free of methionine, isoleucine, and valine) with the aid of 2-bromotoluene-5-sulfonic acid, and 20 gm. of *l*-phenylalanine with the aid of 2,5-dibromobenzenesulfonic acid. If the preparation of *l*-phenylalanine is not desired, *l*-leucine may be obtained more economically by purification of commercial products with the aid of naphthalene- β -sulfonic acid (4).

The methods hitherto employed for the preparation of *l*-phenylalanine are discussed by Baptist and Robson (5). These authors proposed a procedure for the isolation of this amino acid utilizing both its copper salt and its picrolonate, and applied the procedure to hydrolysates of zein and casein. The use of dibromobenzenesulfonic acid would appear to be simpler and more economical with respect to time and material.

The natural *l*-serine has heretofore not been readily available. The classical method for its preparation is that of Fischer and Jacobs (6) who employed alkaloids to resolve *p*-nitrobenzoyl-*dl*-serine. The preparation of *l*-serine from hydrolysates of silk fibroin with the aid of hydroxyazobenzenesulfonic acid was recently reported (2). The detailed procedure is described in the experimental section of this communication. In addition to the 95 gm. of *l*-serine obtained from each kilo of fibroin, 240 gm. of *l*-alanine are isolated as a by-product.

EXPERIMENTAL

The solubility products listed in Table I were obtained by a procedure similar to that reported previously (1), with the following modifications: The amino acids were dissolved in *N* HCl as before, but the sulfonic acids, used as reagents, were dissolved in water. Similarly, in order to determine the solubilities of the amino acid salts formed, each sample was progressively diluted with water instead of *N* HCl. Finally, whenever a sulfonic acid employed as reagent was found to be sparingly soluble in 0.5 *N* HCl at 0°, a sufficient quantity of methyl cellosolve was added to each sample to keep the sulfonic acid in solution.

Preparation of l-Leucine and l-Phenylalanine from Hemoglobin—2 kilos of hemoglobin (Eastman, Technical) were hydrolyzed with 6 liters of sulfuric acid (25 per cent by volume), the sulfuric acid removed exactly with barium hydroxide, and the filtrate and washings concentrated *in*

vacuo to 2.4 liters. The insoluble amino acids which separated during the concentration were removed by filtration. The concentrated hydrolysate was stored at 0° overnight, and an additional crop of insoluble material was obtained. The combined yield was about 500 gm. The filtrate served for the isolation of lysine by the method of Rice (3) and subsequently for the isolation of histidine.

For the isolation of *l*-leucine, 1 kilo of the insoluble amino acid mixture was ground to a fine powder, dissolved in 7.5 liters of boiling water, and decolorized with 250 gm. of acid-washed charcoal. To the hot solution 1 liter of concentrated HCl was added, followed by about 1200 cc. of a solution of 2-bromotoluene-5-sulfonic acid, obtained from 850 gm. of the sodium salt over the barium salt¹ (1). The *l*-leucine salt crystallized from the solution at 0°. The filtrate (Solution A) was saved for the isolation of *l*-phenylalanine. The leucine salt was recrystallized from 2.5 liters of water; yield, 925 gm.

To obtain free leucine, the salt was dissolved in 2 liters of hot water, and a solution of 370 gm. of BaCl₂·2H₂O in 500 cc. of hot water added. The mixture was stored at 0° overnight and the precipitate of barium 2-bromotoluene-5-sulfonate saved for recovery of the reagent.² The filtrate and washings were concentrated *in vacuo* to about 1.5 liters and neutralized with concentrated NH₄OH. The yield of crude leucine, obtained from the mixture at 0°, was about 200 gm. An additional 30 to 40 gm. may be obtained by further concentration of the mother liquor.

For purification, the crude leucine was dissolved in 2 liters of warm concentrated NH₄OH, and the solution decolorized with acid-washed charcoal and placed in an oil bath at 110–120° in order to boil off the ammonia. Concentration of this solution to about 1 liter yielded 160 gm. of sulfur-free leucine, $[\alpha]_D^{20} = +15.5^\circ$ (5 per cent in 21 per cent HCl). Further concentration of the mother liquor yielded an additional 45 gm. of leucine which was submitted to recrystallization.

For the isolation of *l*-phenylalanine, 300 gm. of 2,5-dibromobenzene-sulfonic acid were dissolved with heating in the aforementioned Solution A. The solution was stored overnight at 0°. The yield of *l*-phenylalanine dibromobenzenesulfonate was 300 to 325 gm. The crude salt was recrystallized from 2400 cc. of a solution of 1 part of methyl cellosolve and

¹ The free 2-bromotoluene-5-sulfonic acid is not readily isolated from aqueous solution.

² The barium salt was decomposed with aqueous sulfuric acid. The aqueous solution of the free acid thus obtained was usually colored. Addition of CuCl₂ (analytical reagent), followed by H₂S, decolorized the solution without introducing ash. If desired, the sodium salt may be obtained from the solution by salting-out with NaCl. In this manner 50 to 60 per cent of the starting reagent may be recovered in pure form.

9 parts of water, and the hot solution decolorized with acid-washed charcoal. Yield, about 225 gm. An additional 50 to 60 gm. of salt were recovered by concentrating the mother liquor *in vacuo* to 600 cc.

To 225 gm. of the recrystallized phenylalanine salt were added 250 cc. of water and 50 cc. of pyridine.³ The mixture was heated until a clear solution resulted, and 500 cc. of hot absolute alcohol were added. The mixture was kept at 0° and the phenylalanine obtained washed with alcohol and ether; yield, about 55 gm. The filtrate (Solution B) was worked up as described below. The crude phenylalanine was recrystallized from a mixture of 260 cc. of water, 40 cc. of concentrated NH_4OH , and 600 cc. of alcohol in the manner already described for the recrystallization of leucine. Yield, about 35 gm., $[\alpha]_D^{20} = -34.0^\circ$ (2 per cent in water). An additional 10 gm. of phenylalanine were obtained on concentration of the mother liquor.

Solution B, containing phenylalanine dibromobenzenesulfonate and free dibromobenzenesulfonic acid, was concentrated *in vacuo* to 250 cc., warmed, and 175 cc. of concentrated HCl were added. The precipitate obtained by filtration at 0° was stirred vigorously with 375 cc. of water. About 50 gm. of phenylalanine dibromobenzenesulfonate failed to dissolve, and were kept for resubmission to the pyridine treatment. To the aqueous filtrate, from which the 50 gm. of phenylalanine salt had been removed, concentrated HCl was added, and 75 gm. of dibromobenzenesulfonic acid were recovered.

Sodium 2-Bromotoluene-5-sulfonate (7)—Add 500 gm. of *o*-bromotoluene slowly to 500 cc. of concentrated H_2SO_4 plus 500 cc. of fuming H_2SO_4 (25 per cent SO_3). Keep the temperature below 50°; allow the reaction mixture to stand for 20 minutes and pour into 8 liters of ice. The sodium salt is obtained by salting-out with NaCl , and is recrystallized from water.

2,5-Dibromobenzenesulfonic Acid (8)—Heat 500 gm. of *p*-dibromobenzene for 1 hour on the steam bath with 1 liter of fuming sulfuric acid (25 per cent SO_3). Pour the mixture into 6 liters of ice. Heat, filter the hot solution, and cool the filtrate to 0°. The sulfonic acid which separates is recrystallized from water by addition of concentrated HCl .

Preparation of L-Serine and L-Alanine from Silk Fibroin—Technically degummed Japanese white silk (105 gm.) was boiled for 8 hours with 300 cc. of concentrated HCl , the excess HCl removed *in vacuo* in the usual manner, and the bulk of the residual HCl with lead acetate (analytical reagent). The solution was freed of lead with H_2S , and the clear yellow

³ Decomposition with BaCl_2 is not practicable in this case, since there is not a sufficient difference between the solubilities of the barium and phenylalanine salts of the reagent.

filtrate concentrated *in vacuo* to a syrup. Tyrosine was removed by filtration and the filtrate diluted to about 400 cc.

In this solution 170 gm. of 5-nitronaphthalene-1-sulfonic acid dihydrate were dissolved with heating. The glycine nitronaphthalenesulfonate obtained from the mixture at 0° was recrystallized once from 500 cc. of water, and saved for recovery of the reagent. Yield of recrystallized salt, about 160 gm.

To the hydrolysate remaining after the removal of glycine (volume about 600 cc.) 75 cc. of methyl cellosolve were added and 130 gm. of azobenzene-*p*-sulfonic acid trihydrate were dissolved in the solution with heating. The *l*-alanine salt obtained at 0° was recrystallized once from water⁴ (yield, 104 gm.). From this salt both *l*-alanine and the reagent were recovered in the manner described below. The yield of *l*-alanine was about 24 gm., $[\alpha]_D^{20} = +9.5^\circ$ (9.3 per cent amino acid hydrochloride in water). $\text{NH}_2\text{-N}$, 15.76 per cent.

To the main body of the hydrolysate a solution of 60 gm. of barium acetate (analytical reagent) was added, and the precipitated barium salts removed. The filtrate was freed of barium with a slight excess of sulfuric acid, and concentrated *in vacuo* to a small volume. The solution was diluted to about 400 cc., 50 cc. of methyl cellosolve and 64 gm. of *p*-hydroxyazobenzene-*p'*-sulfonic acid dihydrate were added, and the mixture heated until a clear solution resulted. The yellow *l*-serine *p*-hydroxyazobenzenesulfonate was obtained at 0°. Some free hydroxyazobenzene-sulfonic acid, which is red, may contaminate this precipitate, but it is removed by washing with cold water and by two recrystallizations of the salt from water. Yield of twice recrystallized salt, about 50 gm.

The technique for splitting the amino acid salts and recovering both the amino acid and the reagent is the same in all the instances mentioned here, and will be given in detail only for the case of serine.

To 40 gm. (0.1 mole) of *l*-serine *p*-hydroxyazobenzenesulfonate dissolved in a minimal volume of hot water, a hot solution of 27 gm. (0.1 mole) of barium acetate monohydrate (analytical reagent) was added. The barium sulfonate precipitated immediately but the solution was cooled before filtration. The yellow filtrate, which contained the *l*-serine, was freed of barium with exactly the requisite quantity of sulfuric acid, and acid-washed charcoal added. The water-clear filtrate from the charcoal- BaSO_4 was concentrated to dryness *in vacuo*. The residue was dissolved in water, the solution decolorized with charcoal, if necessary, and absolute alcohol added. The serine thus obtained was recrystallized from water and

⁴ Small amounts of inorganic salts of azobenzenesulfonic acid were removed by filtration of the hot solution.

alcohol. Yield, about 8 gm. The amino acid was recrystallized once more for analysis.

$C_3H_7O_2N$. Calculated.	C 34.3, H 6.7, N 13.3
Found.	" 34.4, " 6.85, " 13.4

The preparations of *l*-serine yielded by this procedure had a specific rotation of $[\alpha]_D^{20} = -6.8^\circ$ (10 per cent in water) and $[\alpha]_D^{20} = +13.9^\circ$ (250 mg. of *l*-serine plus 2.50 cc. of N HCl). Fischer and Jacobs (6) reported $[\alpha]_D^{20} = -6.8^\circ$ and $[\alpha]_D^{20} = +14.3^\circ$. In determination of the above rotations of serine in N HCl, approximately 1 equivalent of acid was employed. Under these conditions, the specific rotation is sensitive to slight variations in the ratio of serine to HCl. Since it is no longer sensitive to minor variations in the acid concentration when 1.2 or more equivalents of HCl are employed, it appears preferable to determine the rotation in $2\ N$ HCl. $[\alpha]_D^{20} = +14.8^\circ$; $[\alpha]_D^{20} = +15.3^\circ$ (10 per cent in $2\ N$ HCl).

For recovery of the hydroxyazobenzenesulfonic acid, the barium salt obtained above was heated in a 1:1 water-methyl cellosolve mixture containing a slight excess of sulfuric acid. After removal of the $BaSO_4$, HCl was added to the warm filtrate. The free sulfonic acid crystallized from the cooled solution. For decomposition of barium 5-nitronaphthalene-1-sulfonate, the salt was suspended in aqueous sulfuric acid.

The recovery of the sulfonic acids from their amino acid salts, calculated as per cent of the sulfonic acids originally employed, was 78 per cent for nitronaphthalenesulfonic acid, 73 per cent for azobenzenesulfonic acid, and 52 per cent for hydroxyazobenzenesulfonic acid.

5-Nitronaphthalene-1-sulfonic Acid—The preparation of this substance has been simplified. The crude glycine salt is obtained from the sulfonation mixture in the manner already described (1). For purification, 200 gm. of this salt are suspended in 4 liters of boiling ethanol, and water added until the salt dissolves. The hot solution is clarified with acid-washed charcoal and the pale yellow glycine salt obtained at 0° is washed with ethanol and ether. After one recrystallization from water, the glycine salt is converted to the free acid over the barium salt.

*Azobenzene-*p*-sulfonic Acid* (9)—Powdered azobenzene (300 gm.) is slowly added to 900 cc. of fuming sulfuric acid (25 per cent SO_3). The temperature should be kept below 50° . After the azobenzene has dissolved, the solution is heated to 70 – 80° for a few minutes, cooled to about 50° , and poured into 5 liters of ice. 1 liter of concentrated HCl is added, the mixture cooled to 0° , and the sulfonic acid which separates is recrystallized from water and HCl.

*p-Hydroxyazobenzene-*p'*-sulfonic Acid*—The potassium salt is prepared in the manner described by Griess (10). It is converted to the free acid over the barium salt.

SUMMARY

On the basis of the solubility products of their amino acid salts, several sulfonic acids derived from diphenylamine, anthraquinone, and azobenzene have been found to be of potential value for the isolation, purification, and determination of amino acids. The utilization of sulfonic acids for the preparation of amino acids is exemplified by the procedures described for the isolation of *l*-phenylalanine and *l*-leucine from hydrolysates of hemoglobin, and of *l*-serine and *l*-alanine from hydrolysates of silk fibroin.

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THE VERATRINE ALKALOIDS

XIII. THE DEHYDROGENATION OF PROTOVERATRINE

By LYMAN C. CRAIG AND WALTER A. JACOBS

(From the Laboratories of The Rockefeller Institute for Medical Research)

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In our further study of the chemistry of the veratrine alkaloids it was of interest to apply to other alkaloids of *Veratrum album* methods similar to those which have been reported in the case of cevine and, more recently, of jervine (1). This has now been extended to the alkaloid protoveratrine, which was first isolated by Salzberger (2) and to which more recently the formula $C_{40}H_{68}O_{14}N$ has been given by Poethke (3). According to the latter this alkaloid yields on saponification acetic acid, *l*-methylethylacetic acid, methylethylglycolic acid, and an amorphous alkamine, protoverine, $C_{28}H_{46}O_{10}N$. Although a relationship in structure of this alkamine and its companion alkamine germinine, $C_{26}H_{44}O_8N$ (3), to cevine, $C_{27}H_{43}O_8N$, has been suspected on general considerations, this has not as yet been supported by any direct evidence. Some such evidence we have now been able to obtain by a study of the dehydrogenation of protoveratrine.

Since the protoverine of Poethke is difficult to purify, we have dehydrogenated protoveratrine itself with selenium. The resulting reaction products yielded a large acid fraction from which it was possible to separate by fractional distillation acetic acid, methylethylacetic acid, and methylethylglycolic acid, thus confirming the observations on saponification by Poethke. From the volatile basic fraction it was possible to separate in relatively small amount a dimethylpyridine, which from the melting point of its picrate (171-174°) appeared to be 2,5-dimethylpyridine (4). A second volatile base was obtained in larger amount which agreed in properties both as the free base and as the picrate with the properties which we have recorded for the 2-ethyl-5-methylpyridine (5) obtained from cevine and from jervine. In a higher fraction an oxygen-containing base was contained which was isolated as the *picrate*. Analysis of the latter indicated a formulation $C_8H_9ON \cdot C_6H_5O_7N_3$. The undistilled residue which resulted from the dehydrogenation yielded a relatively small high boiling fraction. From the latter a crystalline fraction was in turn obtained but in an amount too small for recrystallization to constant melting point. However, analysis, absorption spectrum measurements, and general properties indicated impure cevanthrol, $C_{17}H_{16}O$. Its absorption spectrum curve,¹ together with that of cevanthrol, which hitherto has not been pub-

¹ The absorption spectrum measurements were kindly carried out by Dr. G. I. Lavin of the Rockefeller Institute.

lished, is given in Fig. 1. It can readily be seen from the curves that the type of absorption is similar to that shown by cevanthrol, although the extinction coefficients are uniformly lower, a difference which may well have been due to impurity. Finally, from the non-volatile basic fraction a picrate was obtained in very small amount which when analyzed appeared to be cevanthridine picrate. Although the melting point was somewhat low, it gave no depression when mixed with cevanthridine picrate.

From these data there can be little doubt that the alkamine protoverine has the same type of ring structure as has cevine.

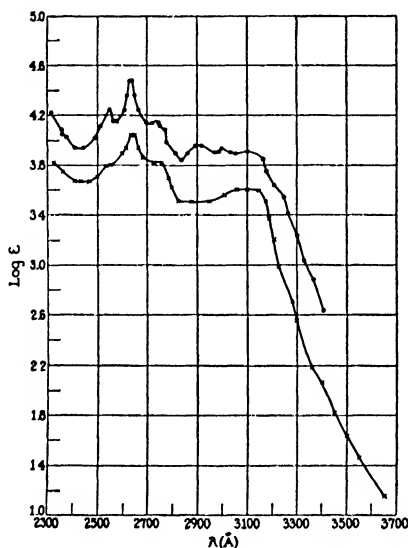


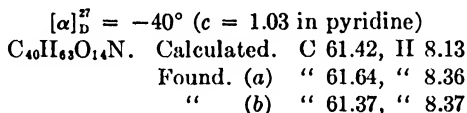
FIG. 1. Absorption spectrum curves determined in ethyl alcohol solution. ○ -- cevanthrol; × = compound from protoveratrine.

EXPERIMENTAL

Protoveratrine—Ground commercial roots of *Veratrum album* were extracted at room temperature as follows: 2 kilo portions were thoroughly mixed with 7 liters of benzene and stirred with a mixture of 100 cc. of ammonia (sp. gr. 0.9) and 1 liter of H₂O. The next day the solvent was filtered and the moist solid was pressed dry. The latter was reextracted with an additional 5 liters of benzene. This was finally followed by a third extraction with 5 liters, which was used directly for the first extraction of a succeeding portion of root. The first two extracts, which had been obtained from a total of 8 kilos of roots, were concentrated *in vacuo* to 3 liters. The clear, dark colored solution was then extracted six times with

1 liter portions of 5 per cent acetic acid. The acid extracts were shaken with 2 liters of benzene and made alkaline with excess 25 per cent NaOH. The extraction with repeated portions of benzene was accomplished as quickly as possible to avoid crystallization of the alkaloid. The benzene extract was washed with water several times, during which a small amount of sparingly soluble alkaloid material (protoveratridine) remained suspended in the aqueous phase. The benzene layer was dried and concentrated to dryness. The resinous residue was dissolved in about 500 cc. of dry ether. Crystallization promptly occurred, and after 18 hours the solid was collected with ether. The yield of this fraction from 8 kilos of root averaged 9.2 gm. This was followed soon by a second fraction from the mother liquor which averaged nearly 4 gm. After many weeks an appreciable third fraction of more soluble alkaloids gradually crystallized. The first fraction consisted essentially of protoveratrine.

For recrystallization the first fraction was suspended in about 10 parts of hot 95 per cent alcohol and an excess of acetic acid was added. On addition of a slight excess of ammonia the alkaloid rapidly separated as a crystalline powder which discolored above 270° and gradually decomposed at 273–276° (uncorrected). On concentration of the mother liquor *in vacuo* a second appreciable crop was obtained.



Dehydrogenation of Protoveratrine—A mixture of 16.6 gm. of protoveratrine and 60 gm. of selenium was heated in a flask after the air was replaced by nitrogen at 340° for 2 hours. 10.5 gm. of oil distilled over into a chilled receiver. The distillate was treated with a little more than 1 equivalent of 1:1 HCl (on the basis of the original alkaloid) and extracted with ether. The ether layer which contained acid and neutral material was set aside to be treated as described below. The aqueous layer was made strongly alkaline with solid KOH and the liberated bases were extracted with a little ether. Treatment of the alkaline aqueous layer with CO₂ did not liberate any phenolic material.

The ether solution of bases was dried over K₂CO₃, concentrated somewhat, and placed in a fractionation apparatus with a fractionating column 10 cm. in length. The record of the fractionation is given in Table I. Each fraction contained approximately 60 mg. of oil.

The analytical data of Fraction 1 suggested a picoline. However, a picrate crystallized readily from acetone and gave analytical data which suggested a dimethylpyridine. After two recrystallizations from acetone

yellow needles were obtained which melted at 171–174°. 2,5-Dimethylpyridine picrate has been reported to melt at this point (4).

$C_7H_9N \cdot C_6H_3O_7N_3$. Calculated, C 46.41, H 3.60; found, C 46.65, H 3.51

Fractions 3, 4, 5, and 6 did not show much variation in boiling point or refractive index. Both were in good agreement with the figures for 2-ethyl-5-methylpyridine obtained from the zinc dust distillation of cevine (5). The analytical data of Fraction 5 also approached the calculated figures for $C_8H_{11}N$; viz., C 79.27, H 9.15. It formed a picrate from acetone solution which melted sharply at 144–145° and agreed in all properties with the picrate of 2-ethyl-5-methylpyridine. The mixture showed no melting point depression.

$C_8H_{11}N \cdot C_6H_3O_7N_3$. Calculated, C 47.98, H 4.03; found, C 48.35, H 4.06

TABLE I
Fractionation of Volatile Bases

Fraction No.	Bath temperature	Column temperature	Pressure	Micro b.p.	n_D^{25}	Analysis	
						C	H
	°C.	°C.	mm.	°C.		per cent	per cent
1	95	63	40	147	1.4942	76.68	7.81
2	95	65	36	156	1.4952		
3	95	67	29	167	1.4952		
4	97	68	29	171	1.4950		
5	97	65	20	171	1.4950	78.88	9.15
6	103	65	10	173	1.4956		
7	170	130	10	202	1.5152		
8	200	130	2	220	1.5245	70.50	8.05

Fraction 8 with 40 mg. of picric acid yielded 12 mg. of compact rhombic crystals from acetone which melted at 114–117°.

$C_8H_9ON \cdot C_6H_3O_7N_3$. Calculated, C 46.14, H 3.30; found, C 46.14, H 3.53

Recrystallization of this material gave crystals which melted at 138–145°. This result combined with the analytical data of Fraction 8 itself makes the experience with this alkaloid similar to that encountered with cevine. Although the analytical data of Fraction 8 indicate a formulation of $C_8H_{11}ON$ (calculated, C 70.02, H 8.08), the picrate prepared from it indicates the presence of a small fraction containing less hydrogen. In our previous experience with cevine it was found that a crystalline picrate could not be obtained from the $C_8H_{11}ON$ base (6).

The above ether extract, which contained the acid and neutral fractions, was in turn extracted with NaOH solution. The neutral fraction which remained in the ether did not yield anything of promise and appeared to

consist mostly of selenium derivatives. The aqueous alkaline extract was saturated with CO_2 and investigated for any phenolic products. This proved to be negative. The aqueous layer was then acidified with an excess of dilute HCl and exhaustively extracted with ether. The combined ether extract after drying over MgSO_4 was concentrated under a fractionating column. An aliquot of the oily residue of mixed acids was fractionated in a fractionating apparatus with a 10 cm. column, as given in Table II. Each fraction contained approximately 100 mg. Fraction 13 was crystalline.

Fraction 3 was found to be practically pure acetic acid ($\text{C}_2\text{H}_4\text{O}_2$, calculated, C 39.99, H 6.70). The boiling point, melting point, and refractive

TABLE II
Fractionation of Acidic Volatile Material

The temperature of the bath was 100° in all cases.

Fraction No.	Column temperature	Pressure	Micro b p. (760 mm)	M p	n_D^{25}	Analysis	
						C	H
	$^\circ\text{C}$.	mm.	$^\circ\text{C}$.	$^\circ\text{C}$		per cent	per cent
1	55	120	115				
2	65	100	115.5	14-15.5			
3	65	70	116	15-16	1.3728	40.00	6.73
4	65	33	118				
5	65	30	124				
6	65	24	157				
7	65	21	173				
8	65	19	174				
9	65	19	174				
10	65	18	174		1.4059	58.80	9.75
11	65	15	174				
12	65	5	174				
13	65	0.2					

index were also in excellent agreement. Fractions 1, 2, and 4, from the data above, appeared to consist essentially of this acid.

The boiling points of Fractions 7 to 12 closely approximated the constant of isovaleric acid. Their identity was substantiated by the refractive index (of Fraction 10), smell, and the analytical data ($\text{C}_6\text{H}_{10}\text{O}_2$, calculated, C 58.77, H 9.87).

The final fraction (No. 13) was crystalline but still contained a little oil. However, the crystals did not entirely melt until a temperature of 68° was reached. Upon recrystallization from a mixture of ether and isopentane felted needles were obtained which melted at $71-73^\circ$ (Poethke reported $72.5-73^\circ$).

$\text{C}_8\text{H}_{10}\text{O}_2$. Calculated, C 50.81, H 8.54; found, C 51.12, H 8.45

The non-volatile residue from the dehydrogenation was powdered and exhaustively extracted with ether. Evaporation of the combined ether extracts gave a residue of only 0.6 gm. It was redissolved in ether and extracted with 10 per cent HCl. The ether layer was separated from the usual tarry precipitate and dried. The acid layer was set aside to be treated as described below. The ether solution yielded a residue of 0.2 gm. For fractionation it was placed in a micro fractionating apparatus with a column 5 cm. in length. Several small fractions were collected up to an oil bath temperature of 230° and under 5 mm. pressure. They appeared, however, to be of hydrocarbon character and were not sufficient in amount to permit of proper fractionation. However, a later fraction of 50 mg. of semicrystalline material contaminated with selenium distilled up to 240°. The selenium was removed with bone-black in ether solution. The filtrate yielded a residue which was dissolved in a little benzene. Upon cooling and seeding with cevanthrol 23 mg. of crystalline material separated which did not exhibit a sharp melting point. After recrystallization 11 mg. of leaflets were obtained which melted at 168–175°.

$C_{17}H_{16}O$. Calculated, C 86.39, H 6.83; found, C 85.92, H 7.29

Obviously the analytical data and melting point are not entirely satisfactory (cevanthrol melts at 195–196°), but there was not sufficient material for further recrystallization after the analysis and absorption spectra measurements.

The above acid layer with the suspended tarry salts of basic products was extracted with chloroform. The chloroform, which dissolved all the precipitated tar, was extracted with 10 per cent NaOH and dried over K_2CO_3 . Evaporation of the extract gave a residue of 250 mg. Upon distillation under 0.2 mm. pressure only a trace distilled up to an oil bath temperature of 200°. However, above this up to 255°, 130 mg. of distillate were collected. This could not be directly crystallized. When mixed with 70 mg. of picric acid in acetone solution, 20 mg. of crystalline material were obtained which melted at 235–245°. This melting point was not raised on recrystallization and is approximately 5° lower than that of cevanthridine picrate. A mixed melting point, however, showed no depression.

$C_{25}H_{17}N \cdot C_6H_3O_7N$. Calculated, C 65.24, H 5.30; found, C 64.92, H 5.56

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A COMPARISON OF ERYTHROCYTE SEDIMENTATION RATES AND ELECTROPHORETIC PATTERNS OF NORMAL AND PATHOLOGICAL HUMAN BLOOD

By THEODORE SHEDLOVSKY, PH.D., AND JOHN SCUDDER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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The suspension stability of blood, as measured by the rate of sedimentation of erythrocytes in plasma, has had wide application in medicine since Fåhræus (1) published the results of his investigations on the subject some twenty years ago. It is well known that the sedimentation rate is increased markedly in many pathological conditions, notably in acute infections. However, although a voluminous literature has grown, dealing with many aspects of this subject, the mechanism responsible for the observed differences in the sedimentation rates of normal and pathological blood is not well understood.

Various workers have called attention to an apparent correlation between increases in the sedimentation rate and corresponding increases in serum globulin or fibrinogen levels. An excellent survey of the subject was made by Ham and Curtis (2), in which they discussed various techniques for measuring sedimentation rates, the influence of erythrocyte size and total volume on the rate of fall, and correlation with fibrinogen levels.

In this paper, we shall report the results of studies on human blood in a number of pathological conditions, obtained by the method of electrophoresis. These results have been correlated with corresponding observations on the erythrocyte sedimentation rates.

Materials and Methods

Sedimentation rate measurements were performed on samples of blood¹ taken into tubes containing a dry mixture of potassium and ammonium oxalates, as recommended by Heller and Paul (3). In some cases heparin (Connaught Laboratories) was used as an anticoagulant instead of the oxalate mixture; about 0.1 mg. of this material was sufficient for 10 cc. of blood. After thorough but gentle mixing the blood was introduced into Rourke-Ernstene (4) tubes, graduated at 2 mm. intervals over a length of 100 mm., containing about 1.25 ml. The tubes were suspended in an accurately vertical position in a glass cylinder of water, the temperature of which

¹ Fasting samples of blood were drawn from the antecubital vein with as little stasis as possible. The sedimentation rate was measured within 2 hours after collection.

remained quite constant, the work being done in a thermostated room at 25°C. Readings of a clock were taken as the erythrocyte boundary came to succeeding graduation marks. The region of uniform rate of fall determined the (uncorrected) sedimentation rate. The partial volume occupied by the cells (hematocrit) was measured after centrifuging the tubes for 30 minutes at 3000 R.P.M. The corrected sedimentation rate² was then obtained with the aid of charts published by Rourke and Ernstone (4).

Electrophoretic studies were carried out in the Tiselius apparatus, using the scanning method of Longworth (5). The technique has been described in detail by Longworth, Shedlovsky, and MacInnes (6). All the determinations were made in diethylbarbiturate buffer solutions at pH 7.8–7.9 and an ionic strength of 0.05, on samples of plasma or serum which had been diluted with 3 volumes of buffer solution against which they were then dialyzed.

RESULTS AND DISCUSSION

In the paper to which we have just referred (6), electrophoretic determinations were made on the plasma or serum from a number of normal individuals, as well as on that of patients suffering from aplastic anemia, rheumatic fever, pneumonia, peritonitis, peritonsillar abscess, acute lymphatic leukemia, lymphogranuloma, obstructive jaundice, lipoid nephrosis, and multiple myeloma. The corresponding sedimentation rates were determined by us, but were not published at that time. The present electrophoretic studies have extended the work to include more normals and cases of tuberculosis, coronary thrombosis, duodenal ulcer, nephritis, arthritis, lymphosarcoma, burns, fractures, and chemically induced shock in treating insanity. The results are summarized in Table I, and some of the corresponding electrophoretic patterns are shown in Figs. 1 and 2. In columns 7 to 10 of the table are given the values for the ratios of α globulin, β globulin, γ globulin, and ϕ (fibrinogen) to albumin, respectively. The concentrations of albumin in the plasma (or serum) appear in column 5, and the albumin:globulin ratios in column 6. The corrected erythrocyte sedimentation rates (E.S.R.) are listed in column 11. The concentrations of the various components were obtained from the areas under the corresponding electrophoretic peaks, such as are shown in Figs. 1 and 2. The values found for the normals in the present series agree closely with those found by Longworth, Shedlovsky, and MacInnes (6). It was pointed out by these authors that the most striking and general change in the electrophoretic patterns of pathological serum is reflected in the α globulin levels, which appear

² The rate of fall of particles in a fluid contained in a tube of finite length is determined in part by the partial volume of the particles, since they fall against a counter-current of the fluid which must rise to replace the space formerly occupied by the particles. The counterforce thus exerted against the falling particles depends on the relative volumes of the particles and of the fluid.

to be significantly increased in cases of various febrile infections. The present results confirm these findings and also indicate that they hold true in cases of

TABLE I
Composition of Normal and Pathological Serum and Plasma
Corrected Erythrocyte Sedimentation Rate

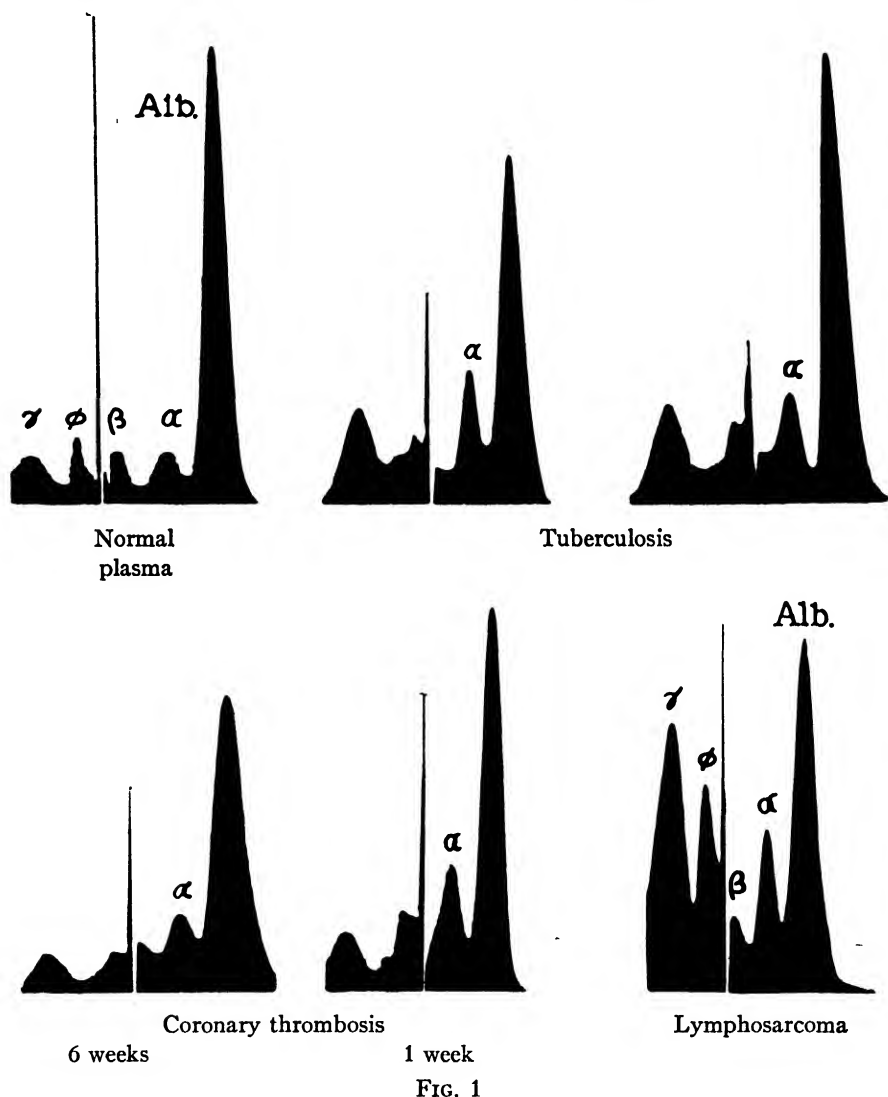
No.	Material	Age	Sex	Albu- min per cent	A/G	α /A	β /A	γ /A	ϕ /A	E S R mm / min	Temp. a m. °F.	Remarks
1	Normal	9	M	3.45	2.27	0.11	0.18	0.15	—	0 10		
2	Normal	40	M	4.06	1.75	0.16	0.25	0.16	—	0.44		
3	Normal	38	F	4.00	2.00	0.14	0.17	0.19	—	0.50		
4	Oligophrenia	38	F	4.66	1.92	0.11	0.18	0.23	0.10	0.40		Phenyl pyruvic acid
5	Psychopathic per- sonality	22	F	5.05	2.00	0.16	0.12	0.22	0.07	0.30	99.6	During metrazol shock
6	Schizophrenia simplex	16	F	4.33	2.00	0.17	0.14	0.19	0.10	0.55	96.0	During insulin shock
7	Fracture of tibia and fibula	72	M	3.92	2.13	0.17	0.16	0.14	0.11	0.70	98.6	3 days after accident
8	Fracture of femur	40	M	4.21	1.39	0.21	0.30	0.21	—	1.40	101.2	2 days after accident
9	Burns, first, sec- ond, and third degree	55	F	3.77	1.04	0.36	0.36	0.25	0.14	2 60	101.0	6 days after accident
10	Duodenal ulcer	58	M	4.55	1.56	0.20	0.25	0.19	—	1.20	98.6	Complicated with hem- orrhage and obstruc- tion
11	Coronary throm- bosis	42	M	3.34	1.07	0.34	0.32	0.27	—	1.40	99.4	1 week after initial attack
12	Coronary throm- bosis	43	M	3.40	1.54	0.19	0.30	0.16	—	1.00	98.4	6 weeks after initial attack
13	Tuberculosis	57	M	4.41	1.11	0.19	0.36	0.35	—	1.80	98.6	Chronic pulmonary
14	Tuberculosis	34	M	3.31	0.90	0.34	0.34	0.43	—	1.30	102.0	Bilateral pulmonary, far advanced
15	Tuberculosis	39	M	2.95	0.83	0.30	0.34	0.57	0.21	1.80	102.5	Bilateral pulmonary, far advanced
16	Tuberculosis	40	M	3.85	1.10	0.17	0.30	0.34	0.10	0.40	100.0	Miliary, tuberculous meningitis
17	Tuberculosis	38	M	3.11	1.10	0.33	0.34	0.56	0.24	2 20	100.0	Bilateral pulmonary, far advanced
18	Neoplastic disease	36	F	3.80	1.01	0.19	0.29	0.51	—	1.20	98.0	X-ray: general skeletal involvement
19	Lymphosarcoma	14	F	3.22	0.70	0.34	0.19	0.91	0.42	1.90	98.0	Febrile Biopsy
20	Chronic nephritis	30	F	3.30	1.33	0.39	0.20	0.26	0.10	1.40	98.4	Toxemia of pregnancy. Post partum
21	Arthritis	18	F	3.77	1.19	0.34	0.25	0.26	—	3.00	101.4	Gonococcus, 9 day his- tory

extensive tissue destruction, as evidenced by the patterns for coronary thrombosis, burns, and fractures³ (Figs. 1 and 2). No significant deviation from normal in either sedimentation rate or electrophoretic patterns was found in the cases (4, 5, 6 of Table I) of chemically induced shock in demented patients.

Various authors have reported that good correlation exists between sedi-

³ Determinations on the plasmas of three normal individuals whose temperatures were raised by artificial fever to 106°F. for 40 minutes showed no increase in α globulin.

mentation rates and fibrinogen levels (2). However, the addition of purified fibrinogen to normal blood fails to increase the rate of sedimentation of eryth-



FIGS. 1 and 2. Electrophoretic patterns on normal human plasma and on plasma from individuals with tissue injury from various causes.

rocytes to the same extent as one observes in pathological blood containing comparable quantities of this protein. In Fig. 3, we have plotted the sedimentation rate (E.S.R.) against corresponding levels of fibrinogen expressed as fibrinogen:albumin ratios (ϕ/A), using the results of Ham and Curtis (2).

It will be observed that only a qualitative correlation exists between these two factors.

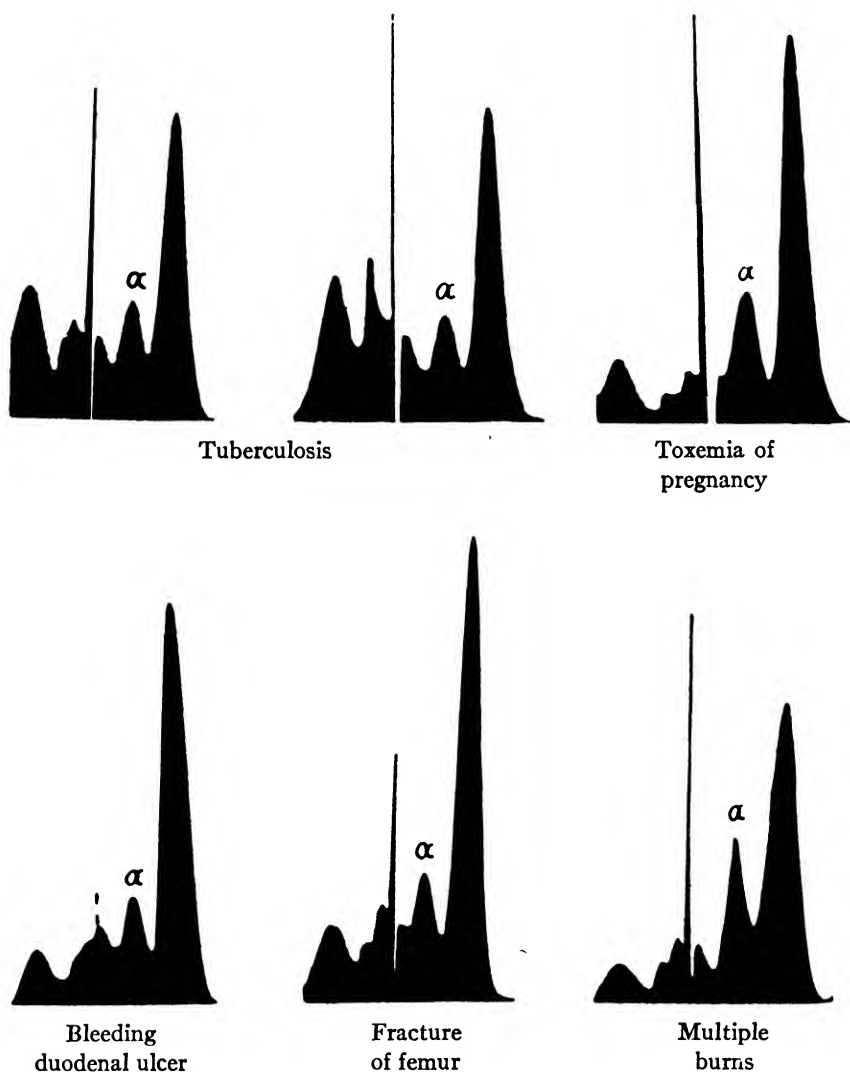


FIG. 2

We have found no satisfactory similar correlation with either β globulin, γ globulin, or albumin:globulin ratios. However, as will be shown below, a significant correlation can be demonstrated between sedimentation rates and the corresponding α globulin present in the blood.

In Fig. 4 are plotted the α globulin levels, expressed as α globulin:albumin

ratios, (α/A), against the corresponding corrected sedimentation rates (E.S.R.). In this graph have been included points corresponding to the results reported by Longworth, Shedlovsky, and MacInnes (6) as well as our more recent determinations. Although it is not possible to draw a smooth curve through the points indicated, any more successfully than in Fig. 3, an in-

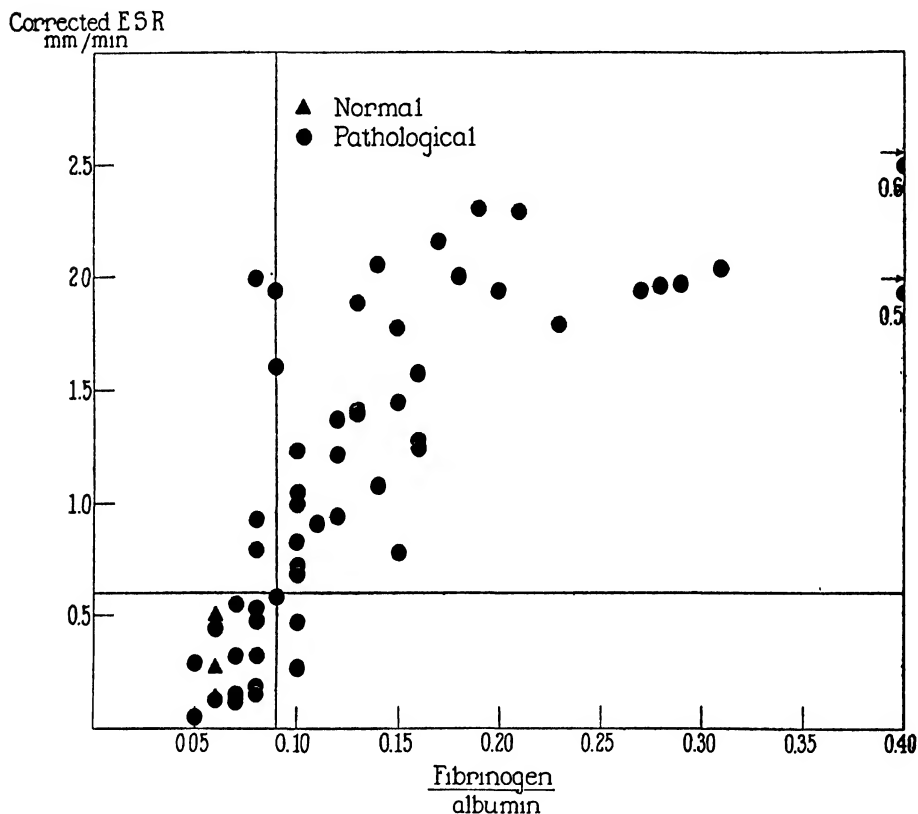


FIG. 3. Variation of corrected erythrocyte sedimentation rates with fibrinogen levels.

teresting relationship appears. By drawing a horizontal line corresponding to the upper limit of normal sedimentation rate and a vertical line corresponding to the upper limit of normal α/A values ($\alpha/A = 0.17$), we find all the points corresponding to the normals, as well as those for nearly all the pathological conditions which yield normal electrophoretic patterns, in the lower left hand quadrant. The other points, corresponding to elevated sedimentation rates, fall in the upper right hand quadrant. If we were to draw another vertical line at about $\alpha/A = 0.20$, all the points in this more restricted upper right

hand quadrant, with one exception, correspond to febrile conditions. The significance of fibrinogen and of α globulin in the mechanism responsible for increased sedimentation rates in blood will be discussed in another paper.

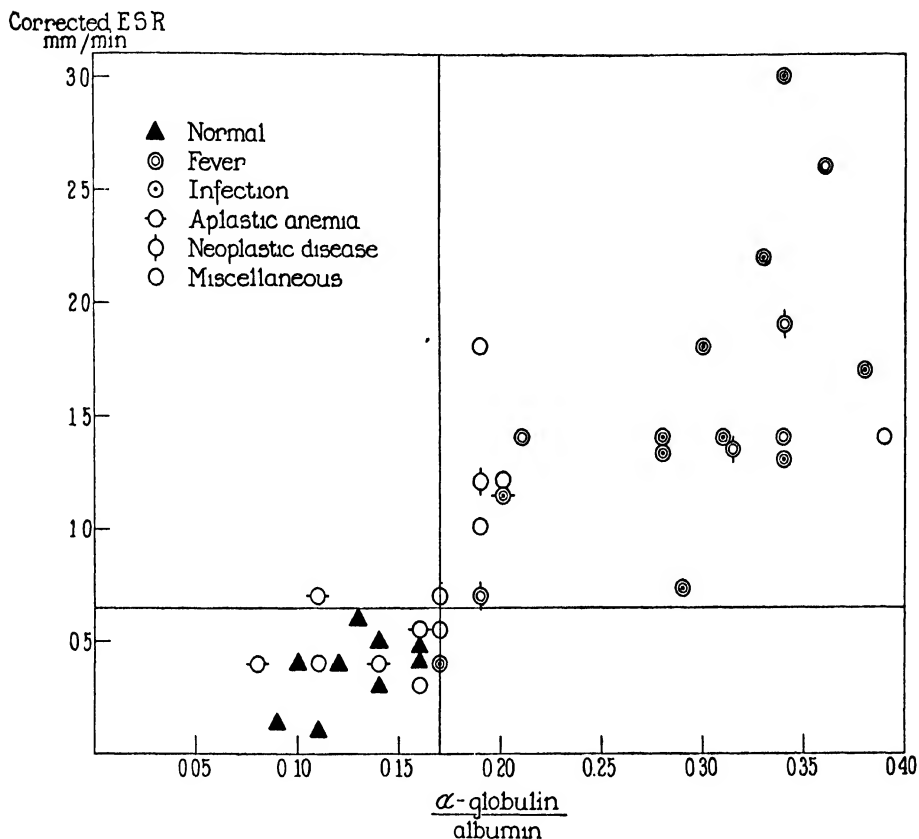


FIG. 4. Variation of corrected erythrocyte sedimentation rates with α globulin levels.

We wish to express our appreciation to the following hospitals for their cooperation: City of New York, Department of Hospitals, Bellevue Hospital, Metropolitan Hospital; Presbyterian Hospital; Rockefeller Hospital; Sloane Hospital for Women; and State of New York, Department of Mental Hygiene, Psychiatric Institute and Hospital.

SUMMARY

Electrophoretic studies and erythrocyte sedimentation rate measurements were carried out on normal and pathological human blood. An increase in α globulin levels appears to take place, as well as an increase in sedimentation

rates, when there is present any considerable inflammation or tissue destruction, irrespective of its cause. A graphic correlation is presented between sedimentation rates and α globulin levels, which is at least as good as a similar correlation involving fibrinogen levels.

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PURIFIED DIPHTHERIA ANTITOXIN IN THE ULTRACENTRIFUGE AND IN THE ELECTROPHORESIS APPARATUS

By ALEXANDRE ROTHEN

(From the Laboratories of The Rockefeller Institute for Medical Research)

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Several preparations of diphtheria antitoxin of different degrees of purity described by Northrop (1) in the preceding article were investigated in the ultracentrifuge and in the electrophoresis apparatus. The main object was to test the homogeneity of the materials and also to obtain a value for the molecular weight of the purified antitoxin.

Ultracentrifuge Studies.—At the time this investigation was started, the only ultracentrifuge studies reported on diphtheria antitoxin were those of Pappenheimer, Lundgren, and Williams (2) and of Paic (3). The molecular weight of antitoxin arrived at by Pappenheimer and coworkers was 184,000, whereas Paic, in a very indirect way, estimated that the size of antitoxin was between that of serum albumin and serum globulin. It soon became evident that the preparations obtained by Northrop with trypsin digestion were homogeneous in the ultracentrifuge, but that the value of the sedimentation constant, $s_{20}^{\text{water}} \simeq 5.5 \times 10^{-13}$ was considerably smaller than the value $s_{20}^{\text{water}} = 7.2 \times 10^{-13}$ found by Pappenheimer *et al.* Since then, Petermann and Pappenheimer (4) obtained by pepsin digestion an antitoxin fraction which was homogeneous in the ultracentrifuge, with a sedimentation constant $s_{20}^{\text{water}} = 5.7 \times 10^{-13}$ but only 33 per cent of the material was specifically precipitated by toxin. Tiselius and Dahl (5) found also very recently that a pepsin-digested fraction, prepared according to Pope, was homogeneous in the ultracentrifuge with a constant of sedimentation $s_{20}^{\text{water}} = 5.4$ to 5.9×10^{-13} . The results which were obtained in the present study are summarized in Table I.

The samples of antitoxin obtained by treatment of the toxin-antitoxin complex with trypsin had different antitoxin titer by the flocculation test. They were also completely precipitated by toxin (Northrop (1), Table VII). However, it is apparent from the table that they all had very nearly the same constant of sedimentation, $s_{20}^{\text{water}} \simeq 5.5 \times 10^{-13}$. The difference between the highest value, 5.7, and the lowest, 5.3, is probably not significant, especially if one considers that the value 5.3 was obtained in an experiment with a high protein concentration. The sedimentation constant for the crystalline material was $s_{20}^{\text{water}} = 5.65 \times 10^{-13}$. It seems probable that the preparations con-

TABLE I
Sedimentation Data

Sample	Northrop corresponding sample	Concentration protein	Medium	pH	\bar{s}_{20}	η_{90}	t	$s_1 \times 10^{13}$	$s_0 \times 10^{13}$
		<i>per cent</i>				<i>millipoise</i>	$^{\circ}\text{C.}$		
1. Trypsin-digested	Fract. 6, Table V	1.1	Phosphate 0.1 M	7.5	1.0070	11.60	27.2	6.00	5.27
2. Trypsin-digested	Fract. 8, Table V	0.5	Phosphate 0.05 M	7.35	1.0043	11.61	11.9	4.34	5.56
3. Trypsin-digested	Fract. 8, Table V	0.3	Phosphate 0.05 M plus 1 per cent $(\text{NH}_4)_2\text{SO}_4$	7.3	1.0107	10.46	18.6	4.85	5.41
4. Trypsin-digested	Fract. 8, Table V	0.15	Phosphate 0.05 M plus 1 per cent $(\text{NH}_4)_2\text{SO}_4$	7.3	1.0107	10.46	17.2	4.92	5.40
5. Trypsin-digested	Fract. 8, Table V	0.65	Phosphate 0.1 M plus 2 per cent $(\text{NH}_4)_2\text{SO}_4$	7.4	1.0258	11.48	1.63	4.38	5.73
6. Trypsin-digested	Fract. 12, Table V	0.2	Phosphate 0.05 M	7.35	1.0043	11.61	18.3	5.28	5.65
7. Trypsin-digested	Fract. 8, Table V	0.16	Acetate 0.1 M	2.9	1.0077	10.40	11.9	4.29	5.56
8. No enzymatic digestion	Fract. 45-55, Table XII	0.5	Phosphate 0.1 M plus 2 per cent $(\text{NH}_4)_2\text{SO}_4$	7.4	1.0390	11.70	24.5	5.75	6.79
9. No enzymatic digestion	Fract. 45-55, Table XII	0.85	Phosphate 0.05 M plus 1 per cent $(\text{NH}_4)_2\text{SO}_4$	7.3	1.0112	10.46	19.5	6.31	6.88
10. No enzymatic digestion	Fract. 45-55, Table VII	0.15	Phosphate 0.05 M plus 1 per cent $(\text{NH}_4)_2\text{SO}_4$	7.3	1.0112	10.46	18.9	6.48	7.18
11. No enzymatic digestion	Fract. 5, Table II	0.28	Phosphate 0.05 M plus 1 per cent $(\text{NH}_4)_2\text{SO}_4$	7.3	1.0112	10.46	18.5	6.27	6.84

TABLE I—*Concluded*

Sample	Northrop corresponding sample	Concentration protein	Medium	pH	δ_{20}	η_{20}	t	$s_t \times 10^{13}$	$s_{20}^0 \times 10^{13}$
		per cent				milli-poise	°C.		
12. Normal plasma globulin fraction	Fract. 45-55, Table XII	0.9	Phosphate 0.05 M plus 1 per cent $(\text{NH}_4)_2\text{SO}_4$	7.3	1.0112	10.46	19.0 ₅	6.20	6.86
13. Normal plasma globulin fraction	Fract. 45-55, Table XII	0.9	Phosphate 0.05 M plus 1 per cent $(\text{NH}_4)_2\text{SO}_4$	7.3	1.0112	10.76	18.6 ₅	6.25	6.98
14. No enzyme toxin-antitoxin complex	Table IX	0.24	Acetic acid 0.05 M	3.6	1.0025	10.15	19.7	$\begin{cases} 5.98 \\ 8.40 \end{cases}$	$\begin{cases} 6.26 \\ 8.80 \end{cases}$

sisted of several proteins of the same molecular size and shape but which reacted with different amounts of toxin.

Experiments performed with antitoxin preparations obtained without trypsin treatment are summarized in the second half of Table I. All preparations, whatever their titer in antitoxin, have the same sedimentation constant, $s_{20}^{\text{water}} \approx 6.8 \times 10^{-13}$. For example, Experiment 12 was carried out with a fraction obtained from normal plasma, the material of Experiment 8 contained only 15 per cent antibodies, whereas the ratio L_f/PN , of the material of Experiment 11, was as high as that found for the purest sample obtained by trypsin digestion (900 L_f/PN). However, the sedimentation constant of the three specimens was the same within experimental error. Some of the sedimentation patterns can be seen in Fig. 1.

It is worth mentioning that Kekwick and Record (6) recently came to the conclusion that there were at least two distinct diphtheria antitoxins, one present in the β -globulin and the other in the γ -globulin fraction of horse serum. They reported that the constant of sedimentation of the β -fraction was 7.18×10^{-13} , whereas that of the γ -fraction was 6.87×10^{-13} in agreement with the value reported here. Experiment 14 was made with the toxin-antitoxin complex dissolved in 0.05 M acetic acid. Two components could be detected in roughly the same amount, but the sedimentation constants corresponded neither to toxin nor antitoxin.

The diffusion constant was determined optically as previously described

(7). The measurements were made at 20° with the same sample, and the same medium used for Experiments 3 and 4 of Table I. The concentration in protein was 0.3 per cent. The diffusion constant was calculated from $D = \frac{S^2}{4\pi t H_{\max}^2}$, where S is the diffusion area in cm^2 , t the time in seconds, and H_{\max} the maximum height of the curve in centimeters. It was found, for this

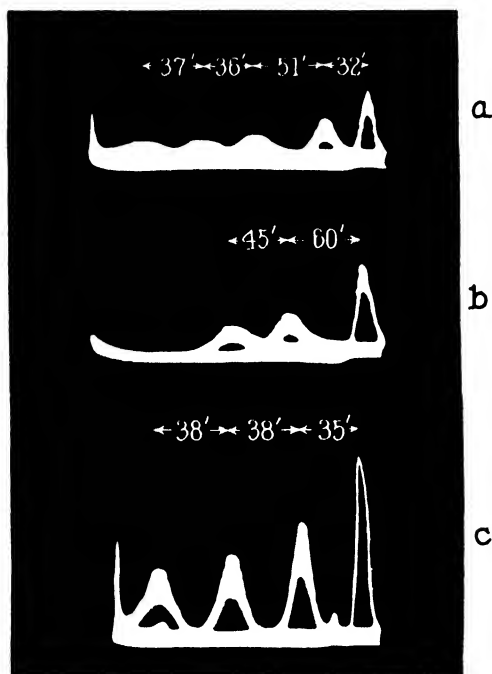


FIG. 1. All patterns obtained during the course of each sedimentation experiment have been superimposed on the same plate. *a*, *b*, and *c* refer to Experiments 2, 3 and 12 respectively (see Table I). A trace of the heavy component $s_{20}^{\text{water}} = 18.0 \times 10^{-13}$ can be seen in Fig. 1 *c*.

particular medium, $D_{20} = 5.56 \times 10^{-7}$ from which the calculated value $D_{20}^{\text{water}} = 5.76 \times 10^{-7}$.

Data have been summarized in Fig. 2 where the values $\frac{1}{H^2}$ are plotted against t . As required by the theory, a straight line is obtained which extrapolated passes through the origin, which shows that no disturbance occurred at the time of formation of the boundary. This had been observed by Longworth (8) who used the linear relationship H against $1/\sqrt{t}$ to represent his results on the diffusion of egg albumin. As seen from the diagram, seven "diffusion

areas" were determined; the relative values of the areas have been indicated inside the patterns, the constancy of the values is satisfactory. Since the ordinates of the diagram represent the $\frac{1}{H^2}$ values, the relative heights H have been indicated for each pattern.

Molecular Weight of the Purified Trypsin-Treated Antitoxin

The molecular weight was calculated from the usual formula $M = \frac{RTs}{D(1 - V\rho)}$ with the numerical values, $s_{20}^{\text{buffer}} = 5.02 \times 10^{-13}$ (Experiments 3 and 4), $D_{20}^{\text{buffer}} = 5.56 \times 10^{-7}$, $V_{20} = 0.749$, $\rho_{20} = 1.0107$, from which $M = 90,500$.

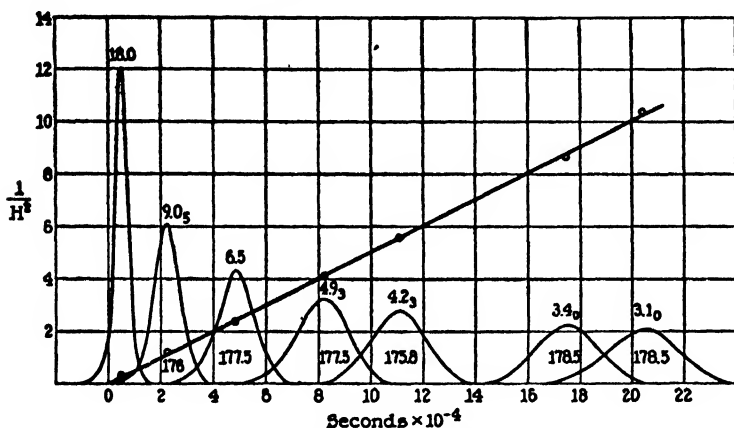


FIG. 2. Diffusion data.

The shape factor $\frac{f}{f_0} = \frac{RT}{6\pi D\eta N} \frac{(4\pi N)^{\frac{1}{3}}}{(3VN)}$ was 1.23.

Petermann and Pappenheimer reported for their pepsin-treated sample $D_{20}^{\text{water}} = 5.8 \times 10^{-7}$, $\frac{f}{f_0} = 1.22$, and $M = 98,000$.

It can then be concluded that the antitoxin molecule obtained by pepsin treatment has the same size and shape as the pure antitoxin obtained by trypsin treatment. The statement of Petermann and Pappenheimer that, "the increase in immunological potency of the digested antitoxin is directly proportional to its decrease in size," does not seem to hold in the case of the trypsin-treated antitoxin since, as mentioned above, the fraction used in Experiment 11 with a sedimentation constant of 6.84×10^{-13} was as active as the pure fraction of Experiment 6 with a sedimentation constant of 5.65×10^{-13} .

Electrophoresis Experiments.—Some of the results obtained can be seen in Fig. 3. Fig. 3 *a* represents the pattern of the descending boundary obtained,

205 minutes after the beginning of electrolysis, with the fraction used for centrifugation Experiment 7 (pH 2.9, specific conductivity 0.01095 at 0°). One component was present with a mobility $u = 4.05 \times 10^{-5}$. Current was reversed for 205 minutes and the pattern appeared as seen in Fig. 3 *a'*. There was no appreciable reversible spreading. Figs. 3*b*, 3*c*, 3*d*, correspond to fractions respectively 6, 8, and 12 of Table V of Northrop's article (1). The samples were dialyzed in the same phosphate buffer pH 7.3, 0.05 M (specific conductivity 0.00336 at 0°). Pictures *b*, *c*, and *d* were taken after electrolyzing for 3 hours, 3 hours, and 2½ hours respectively (8 volts/cm.). After reversal

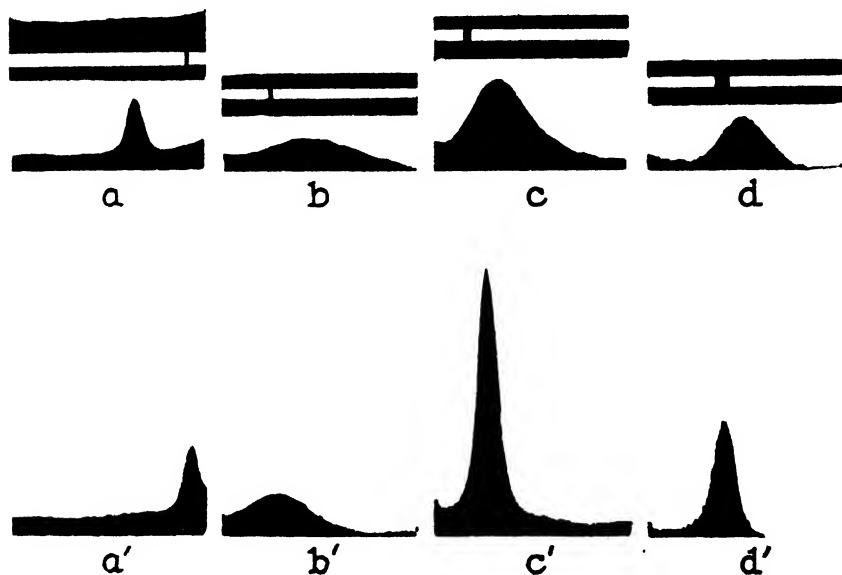


FIG. 3. Electrophoretic patterns of purified trypsin-treated antitoxin.

of the current electrolysis was continued for the same respective times and the appearance of the patterns was as shown in Figs. 3*b'*, 3*c'*, 3*d'*. Only one component could be detected moving very slowly towards the anode with a mobility $u = -0.4 \times 10^{-5}$. There was, however, a considerable amount of reversible spreading in all three samples, especially in No. 6. It has been often suggested that reversible spreading observed during electrophoresis of a protein near its isoelectric point is a measure of its electrical homogeneity. Since, in this case, the amount of reversible spreading was the same in two samples, one of them with constant solubility properties, the question arose whether another cause than heterogeneity could account for the reversible spreading. As suggested by Northrop (1), the phenomenon might be connected with electroendosmosis. Experiments were then made with samples

of fraction 8, one being dialyzed in veronal buffer $m/15$, pH 7.2 (specific conductivity 0.00273 at 0°), the other in veronal buffer $m/15$ plus $m/20$ CaCl_2 , pH 7.1 (specific conductivity 0.00841 at 0°). Both electrophoresis experiments were carried out with a current density of 0.027 amp./cm.² and the periods were so chosen as to make the products $e \times t$ the same in both cases. Results

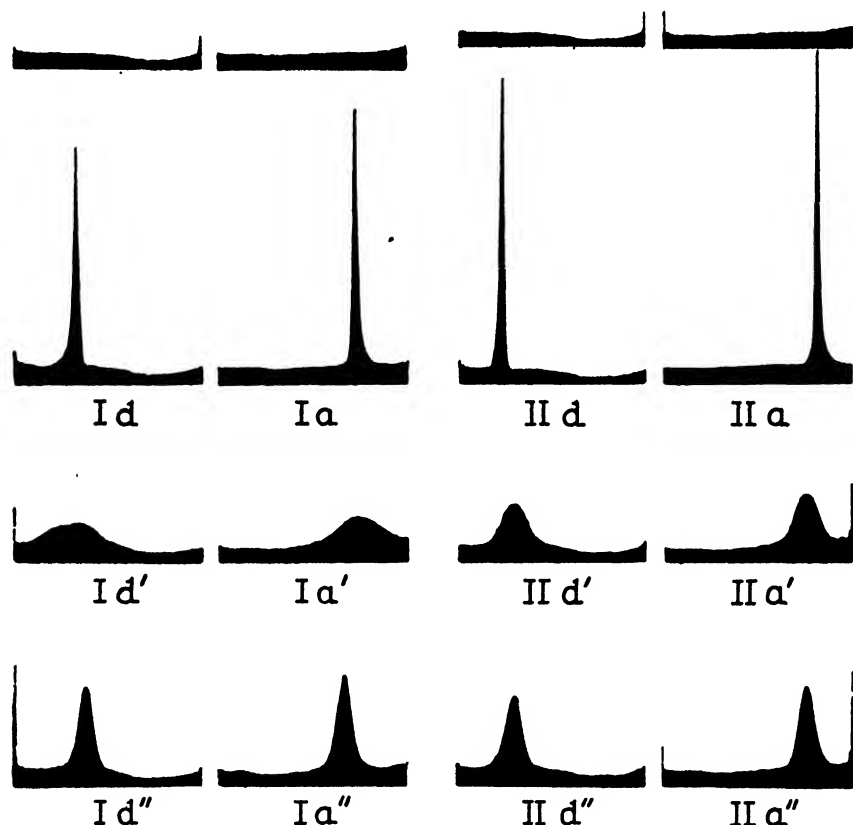


FIG. 4. Electrophoretic patterns of purified trypsin-treated antitoxin, demonstrating the effect of salts on reversible spreading.

can be seen in Fig. 4. I refers to veronal buffer, II refers to veronal plus CaCl_2 buffer, "a" and "d" stand for ascending and descending boundaries, Ia and Id show the boundaries before electrolysis, Ia' and Id' after 85 minutes of electrolysis, and Ia'' and Id'' 87 minutes after reversal of current. The amount of reversible spreading is considerable and the difference in the pattern of the rising and descending boundary is small. IIa and II d are the initial boundaries, IIa' and II d' are the boundaries 240 minutes later, IIa'' and II d'', the boundaries 240 minutes after reversal of the current. The amount of

reversible spreading is small. The presence of CaCl_2 has diminished the spreading.

It can be concluded that reversible spreading in this case is not a measure of the homogeneity of the material.

Some electrophoresis experiments were conducted to find out how different the pattern of an immune plasma would be from that of a normal plasma to which some purified trypsin-treated antibodies had been added. Dilute samples of normal plasma, immune plasma, and normal plasma to which was added the same number of units present in the immune plasma, were dialyzed against the same phosphate buffer, pH 7.3, 0.04 M (specific conductivity 0.00356

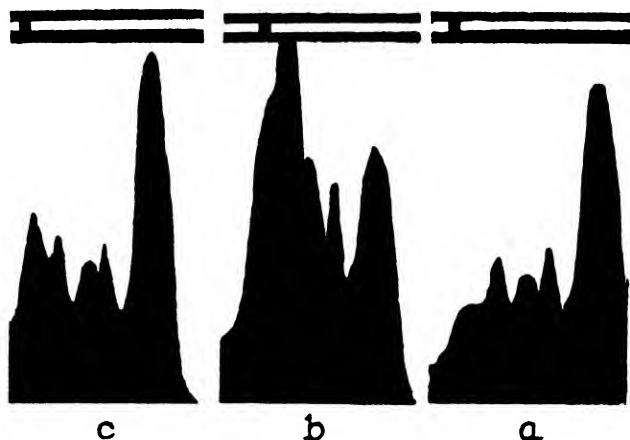


FIG. 5. Electrophoretic patterns of normal plasma, immune plasma, and normal plasma plus purified antibodies.

at 0°). After dialysis, the samples of immune plasma and normal plasma plus antitoxin, contained about 250 units per milliliter. Electrolysis of each of the three samples was carried out with a current density of 0.027 amp./cm.^2 and the appearance of the pattern after $1\frac{1}{2}$ hours can be seen in Fig. 5; *a* corresponds to the normal plasma, *b* to the immune, and *c* to the normal plus purified antitoxin (all descending boundaries). The differences are sharply marked. The addition of purified antitoxin has slightly enhanced the amount of the γ -globulin component of the normal plasma, whereas the pattern of the immune plasma is very different. There is a slight decrease of albumin, as so often noticed with immune plasma, a small increase of α - and β -globulins, and a very large one of γ -globulin, so large, in fact, that it prevented the resolution of the fibrinogen component. The important conclusion to draw is that the large amount of γ -globulin present in the immune plasma contains either antibodies of much lower activity besides the pure antitoxin isolated, or a large amount of inert protein.

Measurement of the area under the different maxima of the patterns permits estimating the concentration of the corresponding components. As can be seen from Table II, the concentration of the γ -globulin fraction plus antitoxin in the immune plasma is 1.6% per cent as compared to 0.6 per cent for the corresponding fraction of normal plasma, plus purified antitoxin, having the same total immunological potency.

It appears from Table II that the large γ -fraction of the immune plasma has an average mobility nearly twice that of the γ -fraction of the normal plasma plus antitoxin. Since the fibrinogen component was not resolved in the pattern of the immune plasma, definite conclusions cannot be made. It is worth mentioning that Tiselius and Dahl (5) found that their immune glob-

TABLE II
Electrophoresis of Dilute Whole Plasma

Protein constituent	Normal plasma		Immune plasma (250 units)/ml.		Normal plasma plus purified antibodies (250 units)/ml.	
	Concen- tration	Mobility	Concen- tration	Mobility	Concen- tration	Mobility
	per cent	$-u \times 10^6$	per cent	$-u \times 10^6$	per cent	$-u \times 10^6$
Total	3.5	—	4.8 ₆	—	3.65	—
Albumin	1.7 ₆	4.9	1.4	4.4	1.65	5.2
α -Globulin	0.40	3.2	0.53	2.9	0.40	3.3
β -Globulin	0.39	2.5	0.78	1.8	0.40	2.6
Fibrinogen	0.48	1.5	0.4 ₆		0.42	1.4
γ -Globulin, antibodies	0.43	0.4	1.6 ₆	0.9	0.64	0.4

ulin fraction with a mobility of 2.0 to 2.4×10^{-6} was gradually transformed, by treatment with pepsin, into a component merging into the γ -fraction.

Ultracentrifuge.—The apparatus previously described was used. The control of the temperature of the rotor has since been greatly improved. Following a suggestion of Dr. MacInnes of these laboratories, the possibility of making sedimentation experiments at low temperature was investigated. A cooling coil connected with a commercial frigidaire unit was installed inside the vacuum chamber. The chamber was insulated by two concentric cylinders of polished chrome-plated copper sheets which were placed between the cooling coil and the wall. Both steel end-plates of the vacuum chamber were insulated by discs, also made of chrome-plated sheets. Temperature of the rotor was measured by bringing the junction of an iron-constantan couple into contact with the rotor. This could be accomplished while the chamber was evacuated because the thermoelement was mounted on a rod which could move vertically through an air-tight bearing. With this arrangement the rotor could be kept at any temperature between -5° and room temperature. In order to facilitate

heat exchange, a pressure of 0.15 mm. of hydrogen was maintained in the chamber for high speed centrifugation. All experiments were made at 57,600 R.P.M. Under these conditions the temperature of the rotor could be kept constant or nearly constant for hours if the temperature of the chamber was about 10° lower than that of the rotor. For instance, the temperature of the rotor during Experiment 12 (Table I) was 18.8° before centrifugation and 19.3° after a 2 hour run at 57,600 R.P.M., an increase of only 0.25° per hour, five times less than under a vacuum of 10^{-5} mm. of Hg.

The rate of sedimentation was determined by the Philpot-Svensson "Schlieren" method. In a few cases measurements were duplicated with the Lamm "scale" method. Both methods gave the same value for the calculated constant of sedimentation within ± 0.2 per cent.

SUMMARY

Ultracentrifugation studies of diphtheria antitoxin showed that:

1. Purified antitoxin of high activity obtained from horse plasma without enzymatic treatment has exactly the same sedimentation constant as the globulin fraction obtained in a similar way from normal horse plasma $s_{20}^{\text{water}} = 6.9 \times 10^{-13}$.

2. Purified antitoxin obtained with trypsin digestion of the toxin-antitoxin complex has a sedimentation constant of $s_{20}^{\text{water}} = 5.5 \pm 0.1 \times 10^{-13}$, a diffusion constant of $D_{20}^{\text{water}} = 5.7 \times 10^{-7}$, and a molecular weight of about 90,000.

Electrophoresis experiments demonstrated that:

1. The trypsin-purified antitoxin has an isoelectric point not far from pH 7.0.
2. The reversible spreading noticed at about pH 7.3 cannot be attributed to heterogeneous preparation.
3. The large increase in the γ -globulin fraction occurring during immunization consists either of antitoxin of various degrees of activity or of some inert protein in addition to the antitoxin.

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THE ISOLATION OF A PROTEIN FROM THE PARS NEURALIS OF THE OX PITUITARY WITH CONSTANT OXYTOMIC, PRESSOR AND DIURESIS-INHIBITING ACTIVITIES¹

BY H. B. VAN DYKE, BACON F. CHOW, R. O. GREEP AND ALEXANDRE ROTHEN

(From the Division of Pharmacology, The Squibb Institute for Medical Research, New Brunswick, and The Laboratory of Physical Chemistry, The Rockefeller Institute for Medical Research, New York)

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Following the discovery of the pressor, oxytocic, and renal effects of posterior pituitary extracts, there has been considerable controversy with regard to the number of substances responsible for these effects. Abel and his collaborators (1, 2) maintained that the actual hormone is a "unitary" substance containing all the activities whereas Dudley (3) early showed that at least partial separation of oxytocic and vasopressor activities could be effected. After Kamm and his colleagues (4) had achieved practically complete separation of these two activities, there was general acceptance of the view that at least two active principles (oxytocic and vasopressor) occur separately in the *pars neuralis*. This view was further strengthened by confirmatory experiments by other methods such as those devised in Stehle's laboratory (5, 6). However, Abel (2) did not withdraw his contention that the true hormone is one substance but did admit that two active and separable principles could be extracted from the gland by appropriately drastic procedures. Rosenfeld's (7) ultracentrifugation of chilled and clarified press-juice of ox posterior lobes also supported the view that oxytocic and vasopressor activities are linked together in a single fairly large molecule and may be liberated from the more complex unit by heating at about pH 4.0.

The experiments reported here are believed to demonstrate that a pure protein of the *pars neuralis* can be isolated containing constant amounts of oxytocic, pressor, and diuresis-inhibiting activities in ratios resembling those found in simple extracts of the gland. In the discussion, possible objections to this belief will be considered with regard to both the physico-chemical (e.g. the possible rôle of adsorption) and the physiological aspects of the problem.

METHODS. *Physico-chemical methods.* All determinations of pH were made by means of a glass electrode and were accurate to ± 0.02 pH. The determinations of nitrogen, except those of table 2 for which a micro-Dumas method was employed, were all made by a micro-Kjeldahl method by which as little as 100 micrograms of N could be determined with an accuracy of ± 2 micrograms. In addition to micro-determination of total sulfur, sulfur

¹ A preliminary report was published in the Proceedings of the American Physiological Society (Amer. J. Physiol., 133: 473, 1941).

distribution (cysteine, cystine, methionine, and sulfate) was investigated by the Kassel and Brand modification of the Baernstein method (8). Anson's method (9) was used to detect sulfhydryl groups (cysteine). Solubility determinations were made at room temperature. The electrophoretic characteristics of the protein were determined in the apparatus of Tiselius (10); the electrophoretic patterns were photographed by the method of Longworth (11). Ultracentrifugation of solutions of the protein was performed in a Bauer and Pickels type (12) of ultracentrifuge driven by an air turbine of the turret type. The rate of sedimentation was calculated from "schlieren" patterns photographed by Philpot's method as modified by Svensson (13). In several experiments ultracentrifugation of solutions of the protein in separation cells was performed.

Biological methods. Estimates of biological activity were all made in terms of U.S.P. reference standard of posterior pituitary powder. Oxytocic activity of various fractions was determined by two methods: the response of the isolated guinea pig uterus or the depressor effect on the fowl's blood pressure. The "physiological" solution used for the isolated uterus was that of van Dyke and Hastings modified to contain 0.75 mM of Ca per liter (14). Furthermore no Mg was added to the fluid since this cation has been found to increase the uterine response to the vasopressor principle (15). In determining oxytocic action by the depressor effect of extracts on the fowl's blood pressure, the method of Coon (16) was largely followed. Dogs and less frequently cats were employed to estimate the vasopressor activity of extracts. Doses were kept small and repeated at sufficiently infrequent intervals to avoid tachyphylaxis. All determinations of blood pressure in the fowl, dog, and cat were made with the glass capsule manometer of Anderson (17). Clotting in cannulas was prevented by the use of a compact modification of Trendelenburg's apparatus (18) to infuse slowly isotonic saline containing 15 mgm. per cent of heparin. To anesthetize cats or chickens 200 mgm. of phenobarbital sodium per kilogram body weight were injected intraperitoneally (cat) or intramuscularly (fowl); dogs were anesthetized by the intraperitoneal injection of 1 ml. per kilogram of 40 per cent alcoholic solution of chlorbutanol as recommended by Kamm and his co-workers (4).

In estimating the inhibition of diuresis in rats we modified Burn's method (19) only to the extent of administering fluid (0.3 per cent NaCl) intraperitoneally instead of by mouth. As a rule either 28 or 32 rats (7 or 8 groups) were used at one time. Half of the groups received the standard and half received the extract to be assayed; several days later the same doses of the same standard or extract were administered in reverse order. Therefore, comparison of the extract and standard was made in the same groups of rats (7 or 8 groups of 4 rats each) as nearly simultaneously as possible.

The melanosome-dispersing action of extracts was compared with U.S.P. reference standard in frogs. For this purpose, normal frogs or frogs hypophysectomized by the method of Teague, Noojin, and Geiling (20) were used.

THE PREPARATION OF THE PURE PROTEIN FROM EXTRACT OF POSTERIOR LOBES OF OXEN. A diagram of the method of preparation is reproduced in figure 1. The initial suspension of freshly dissected posterior lobes obtained from frozen pituitaries contains 1 kgm. of tissue in 9 l. of cold 0.01 *N* H₂SO₄. The pH of the suspending liquid varies little (about ± 0.1 pH) from the value given if different batches are compared. After the mixture has been thoroughly stirred by an electric motor it is allowed to stand in a refrigerator (4°C.) overnight. The separation of the residue and of all subsequent precipitates from supernatants is accomplished by centrifugation. Precipitation of active protein by the addition of 80 grams NaCl to each liter of supernatant adjusted to pH 3.90 (step 2) is also allowed to continue overnight at 4°C.

The last step by which complete purification is achieved is repeated until

solubility is constant. This step is carried out at room temperature which probably is an important variable (20 to 25°C.) affecting solubility. In this final step, constant solubility rather than a solubility of exactly 100 micrograms of N per ml. is sought. In our experience the apparently pure protein may have a solubility as low as 80 micrograms of N per ml.; however, this solubility is constant in a solvent made as exactly as possible like that described in figure 1 in the step next to the last.

The method of extraction is not of high efficiency in terms of the total activity available (about 200,000 units per kg. fresh posterior lobes). The residue

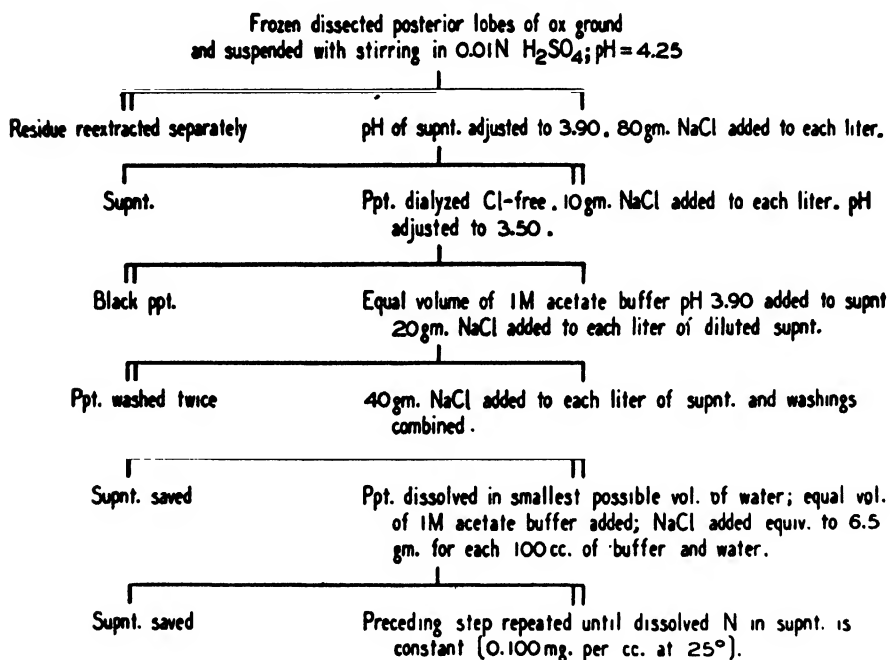


FIG. 1. METHOD OF PREPARING A PURE PROTEIN WITH MULTIPLE ACTIVITIES BY EXTRACTION OF FRESH POSTERIOR PITUITARIES OF OXEN

after initial extraction contains 20 to 25 per cent of total activity which can be removed by boiling a sample of the ground dissected lobes in 0.25 per cent acetic acid. About two-thirds of the 75 to 80 per cent total activity in the supernatant liquid separated from the residue is precipitated by the addition of 80 grams of NaCl to each liter. Therefore, about 50 per cent of total activity is present in the precipitate at the end of the second step. During dialysis of this precipitate activity which apparently is non-protein is also lost; however, we have not attempted to estimate this loss accurately. From 1 kgm. of fresh glands about 700 mgm. of pure protein (>11,000 units) can be isolated apart from subsequent recoveries in supernatants of the last two steps. Samples of the pure protein so far isolated are amorphous.

EVIDENCE THAT THE PROTEIN ISOLATED IS PURE. *Constant solubility.* Northrop and his collaborators (21) in studying crystalline enzymes have applied with great success the solubility test of Sørensen in determining the presence of small amounts of impurity or in demonstrating homogeneity of crystalline enzymes. The solubility characteristics of the protein isolated in the example of figure 1 are shown in figure 2. The solvent used was 0.5 M acetate buffer, pH 3.90, to which 6.5 grams of NaCl were added to each 100 ml. It is clear that by this test there is no evidence that more than one component is present either before the solvent is saturated with the protein or after twenty times the saturating concentration is in suspension. The amounts of protein which have been available have not permitted us to carry out solubility tests in other solvents which should be employed. We have already mentioned that at different times or with other preparations, constant solubility in the same solvent made later might be as low as 0.080 mgm. of N dissolved in each ml. Variables such as

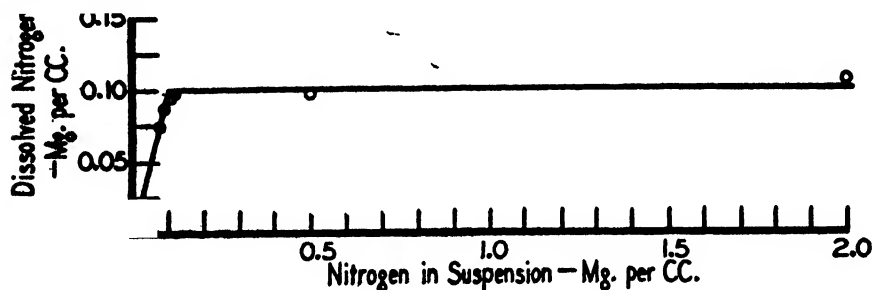


FIG. 2. SOLUBILITY OF THE PURE PROTEIN IN 0.5 M ACETATE BUFFER, pH = 3.90, TO WHICH 6.5 GRAMS NaCl WERE ADDED TO EACH 100 ML.

room temperature or slight changes in the pH of the buffer (e.g., pH 3.95 instead of 3.90) probably explain the variation in absolute amount of protein dissolved.

Electrophoretic homogeneity. A large number of electrophoretic patterns of the pure protein have been photographed in the Tiselius electrophoresis apparatus. In figure 3, mobility per centimeter per second per volt per centimeter has been plotted against pH. The isoelectric point of the protein appears to be about pH 4.8. Examples of patterns photographed by the method of Longworth (11) are shown in figure 4. The upper three patterns were made with three different preparations of pure protein at pH 3.41-3.47. At this pH only, of those used (fig. 3), there appears to be a second protein which is indeed small in comparison with the main component. The oxytocic activity of this minor substance is no greater than that of the main component in terms of nitrogen. At other pHs (e.g., preparation XI-85-G at pH 6.05) there is electrical inhomogeneity but no second protein can be separated. It appears that the minor component at pH 3.4-3.5 is closely related to the main component and possibly is derived from it.

Studies of the protein in the ultracentrifuge. In the ultracentrifuge this material appears to be a single homogeneous protein with a molecular weight of about

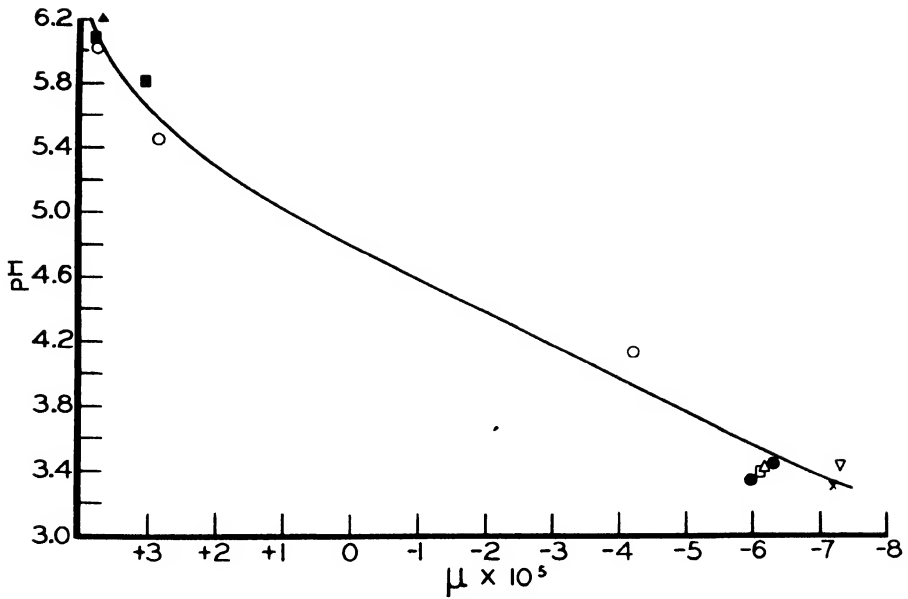


FIG. 3. MOBILITY OF THE PROTEIN IN RELATION TO pH AS DETERMINED IN THE ELECTROPHORESIS APPARATUS OF TISELIUS

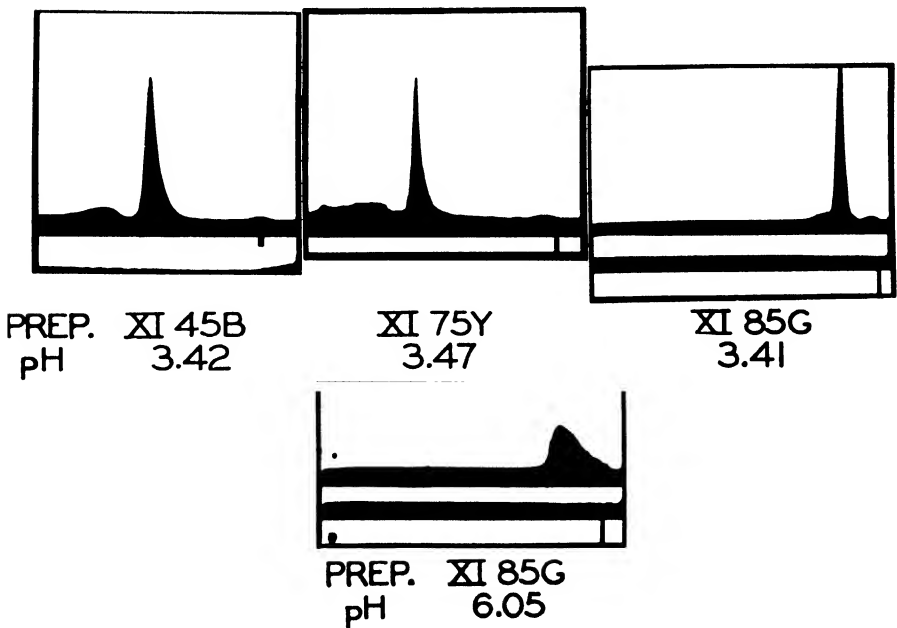


FIG. 4. ELECTROPHORETIC PATTERNS OF THE PROTEIN. SEE TEXT

30,000. The data on which this statement is based are summarized in table 1, which includes a determination of the diffusion coefficient of the first solution. The diffusion constant there given was determined in an apparatus similar to the one used for the electrophoresis experiments in which the Longworth (11)

TABLE 1
Ultracentrifugation studies of pure active protein of the pars neuralis of the ox

PRO- TEIN CON- CEN- TRA- TION	SOLVENT	δ_{20}	η_{sp} (POISES)	t	S_t (EXPERIM)	S_{20}° †	M	REMARKS
per cent				°C.				
1.05	0.1 M acetate buffer, pH 3.30	1.0009	0.01043	7.8	1.98×10^{-13}	2.80×10^{-13}	31,200	Diffusion co- efficient de- termined with same solution, $D_{0.3} = 4.4$ $\times 10^{-7}$; D_{20} (calc.) = 8.5×10^{-7}
0.50	0.34 M NaCl and 0.05 M acetate buf- fer, pH 3.38	1.013*	0.0103*	22.0	2.59×10^{-13}	2.61×10^{-13}	29,700	Not com- pletely ho- mogeneous
0.77	0.17 M NaCl and 0.02 M acetate buf- fer, pH 3.84	1.0055	0.01026	17.6	2.37×10^{-14}	2.61×10^{-13}	29,700	
1.10	0.17 M NaCl and 0.05 M acetate buf- fer, pH 3.35	1.0059	0.01028	18.7	2.42×10^{-14}	2.61×10^{-13}	29,700	Also used for separation expt.
0.19	0.17 M NaCl and 0.1 M acetate buf- fer, pH 3.35	1.0009	0.01024	18.2	2.16×10^{-13}	2.38×10^{-13}		Inaccurate. Boundaries blurred and gradient too low

* Estimated.

† Calculated constant of sedimentation in water at 20°. 0.749 was assumed to be the specific volume at 20°. $\frac{f}{f_0} = 1.15$.

scanning device was employed to obtain the different patterns. At the beginning of the experiment the boundary was slowly pushed into the middle of the Tiselius cell by a compensating device, and the solution was allowed to diffuse into the acetate buffer against which it had been dialyzed. The constant was determined by the formula

$$D = \frac{S^2}{4\pi t l^2 m c}$$

where S is the diffusion area in cm^2 , t the time in seconds, H_{\max} the maximum height of the curve in cm.

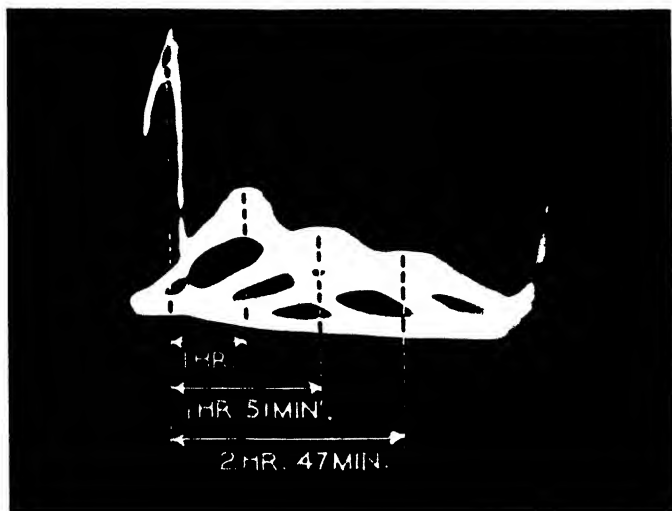


FIG. 5

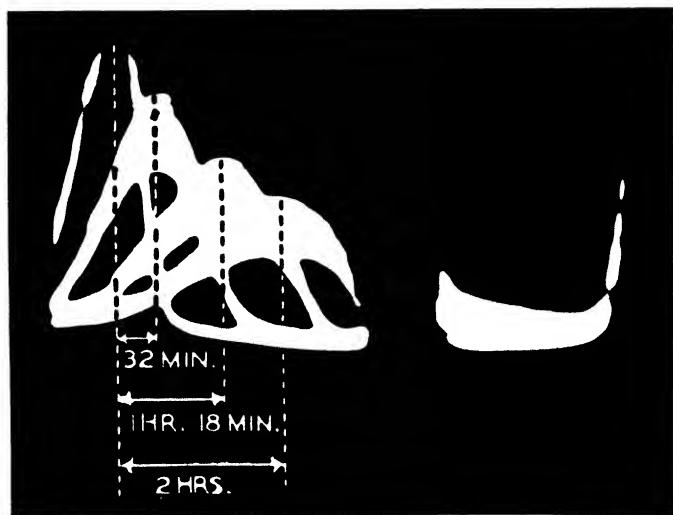


FIG. 6

FIGS. 5 AND 6. SEDIMENTATION PATTERNS OF THE PROTEIN. SEPARATION CELL USED IN OBTAINING PATTERNS OF FIG. 6

Examples of sedimentation patterns are reproduced in figures 5 and 6. Homogeneity is shown by the symmetry of the curves and by the absence of significant displacement of the base line. The specific volume of the protein is assumed to

be 0.749 leading to the calculation of the molecular weight from the usual formula

$$M = \frac{RTs}{D(1 - V\rho)} \approx 30,000$$

where s is the sedimentation constant, D the diffusion constant, V the specific volume of the protein, ρ the density of the solvent, R the gas constant, and T the absolute temperature. The ratio $\frac{f}{f_0} = 1.15$ corresponds to the average value found for most proteins. It means that the molecule can be considered an oblong ellipsoid whose two axes have a maximum ratio of 3.5 provided that there is no solvation.

TABLE 2

Some chemical and physical properties of pure active protein of the posterior lobe of the ox

Elementary analysis:	per cent
Carbon	48.64
Hydrogen	6.63
Nitrogen	16.32
Amino nitrogen	0.054
Phosphorus	0.027
Sulfur	4.89
Chlorine	0.02
Ash	0.58
Oxygen (by difference)	22.80
Distribution of sulfur:	
Cysteine (nitroprusside test)	0
Cystine	4.3
Methionine	?
Sulfate	0.1-0.4
Molecular weight	30,000
Isoelectric point	4.8-4.9

Other properties of the protein. Elementary analysis of the protein yielded interesting results which are assembled in table 2. If one assumes that there is present only one free amino group per molecule, the calculated molecular weight would be about 26,000, a figure in fair agreement with that obtained from the sedimentation constant during ultracentrifugation. The low phosphorus content indicates that the protein is not a nucleoprotein. The high percentage of sulfur led to an investigation of the distribution of this element. In Anson's nitro-prusside qualitative test for free sulfhydryl groups (9), the protein is denatured by guanidine. This test was negative for as much as 2 mgm. of the posterior lobe protein but was positive for 0.085 mgm. of egg albumin. It was therefore concluded that the molecule contains no cysteine. Cysteine-cystine, methionine, and sulfate were all determined by the Kassel-Brand modification of Baernstein's method (8). Although samples for analysis were small, the results indicated that methionine probably is not present and that, in view

of the negative results in testing for —SH groups, nearly all the sulfur is present as cystine sulfur.

THE BIOLOGICAL ACTIVITY OF THE PROTEIN. As many methods of biological assay as appeared to have quantitative value were employed. An especially large number of assays was made of preparation XI-153. Therefore, it seemed best to present these results in some detail to give the reader an idea of the variability encountered by different methods. All assays were performed "blindly," i.e., the concentration of active substance was known to the assayist only to the extent that it fell within broad limits. The U.S.P. reference standard was used in all cases.

On the basis of the experiment of table 3, it is believed that about 20 micrograms of N of the protein are equivalent to 1 mgm. of U.S.P. reference standard

TABLE 3

The potency of preparation XI-153 in inhibiting water-diuresis in the rat

SUBSTANCE INJECTED	DOSE		NUMBER OF RATS	T = AVERAGE TIME REQUIRED FOR EXCRETION OF 50 PER CENT OF URINE	RATIO OF POTENCY	
	U.S.P. unit per kgm.	Micrograms N per kgm.			$\frac{T_C}{T_B}$	$\frac{T_C - T_A}{T_B - T_A}$
A' Control.....			32	62.3		
B U.S.P. Stand.....	0.01		32	118.5	0.94	0.88
C Post. lobe protein.....		0.08	32	111.8		
B U.S.P. Stand.....	0.01		32	112.6	1.03	1.07
C Post. lobe protein.....		0.10	32	115.8		
B U.S.P. Stand.....	0.01		32	109.0	1.13	1.31
C Post. lobe protein.....		0.12	32	122.8		
A'' Control.....			32	66.0		

powder in diuresis-inhibiting action. The same group of 32 rats was used in all the experiments. These were divided into subgroups of 4 each after the technique of Burn (19). The rats were all males weighing about 200 grams. Fluid was not administered by stomach tube as Burn recommended but by the intraperitoneal injection of 50 cc. of 0.3 per cent NaCl per kilogram body weight just before the extract was injected subcutaneously. A given comparison, such as that of the standard (0.01 unit per kilogram) with the protein was performed at an interval of 3 or 4 days so that 4 subgroups receives standard or protein each time with reversal of the groups used for a particular comparison. There was considerable variation in the time of inhibition caused by the standard administered at only one dose level. However, the comparison with the protein led to consistent results. Less elaborate comparisons were made with 4 other pure preparations of the protein. In no case was the apparent potency of the protein represented by a value 20 per cent less than the value given in table 4.

Assays of preparation XI-153 by various methods are summarized in table 4. With the exception of the effect on melanosome-dispersion in the frog, the results indicate that the oxytocic (isolated guinea-pig uterus or fowl blood-pressure), pressor (blood-pressure of cat or dog) or diuresis-inhibiting activity of the preparation is represented by about 10 micrograms of N per U.S.P. unit. In our experience assays of pressor activity are often less reproducible than the results with preparation XI-153 indicate. Pressor activity of other preparations of pure protein in some assays was represented by as much as 12.5 micrograms of N equivalent to 1 U.S.P. unit.

Melanosome-dispersion was studied in normal or hypophysectomized frogs with three different pure preparations. The results were consistent and indicated, as shown in table 4, that U.S.P. extract is 2000 times as potent as our *pars neuralis* protein when this activity is tested in terms of oxytocic-vasopressor units. In the particular experiment cited, the solution of U.S.P. extract (after removal of substances insoluble in the dilute acetic acid) and the solution of

TABLE 4

Biological activity of pure active protein of the posterior lobe of the ox (preparation XI-153)
(No significant deviations were encountered in the assay of four other preparations)

METHOD OF ASSAY	NUMBER OF ASSAYS	AVERAGE AND S.E. OF MICROGRAMS PROTEIN NITROGEN EQUIVALENT TO ONE U.S.P. UNIT
Isolated guinea pig uterus.....	11	9.3 \pm 0.5
Blood-pressure of fowl.....	26	10.4 \pm 0.4
Blood-pressure of cat.....	5	10.4 \pm 0.4
Blood-pressure of dog.....	2	9.3
Diuresis-inhibition in rat.....		10.0*
Melanosome-dispersion in frog.....		20,000

* See table 3 and text.

pars neuralis protein were adjusted to pH 10.5, placed in a boiling water bath for 4 minutes, chilled, and acidified to pH 5.0.² In hypophysectomized frogs the threshold dose of alkali-treated filtered acid extract of U.S.P. powder was found to be about 0.00025 unit per frog.

An international (oxytocic) unit of U.S.P. reference powder is represented by the extract of 0.5 mgm. of powder. Authors often fail to emphasize that the final extract contains far less organic material than that probably contained in the original powder. For example, we commonly make standard extract for assay equivalent to 2 mgm. (4 units) U.S.P. powder per ml. The amount of nitrogen per U.S.P. unit in this solution is 20.3 micrograms. The figure may be higher in commercial posterior pituitary extracts (e.g., 35 micrograms N per unit). Commercial samples of Pitocin and Pitressin vary widely in the amount of nitrogen per unit; the lowest figures found by us were 4.6 micrograms of N

* We owe to Dr. E. M. K. Geiling the suggestion that melanosome-dispersing activity could be more convincingly evaluated if the material were first treated with alkali.

for 1 unit of Pitocin and 3.9 micrograms of N for 1 unit of Pitressin determined from solutions of either extract as marketed commercially. Commercial Pitocin was found to contain very little melanosome-dispersing activity (0.2 per cent that of U.S.P. extract per oxytocic unit) whereas Pitressin had perhaps 50 per cent the activity of U.S.P. extract in this respect. Both comparisons were based on oxytocic or pressor units.

EVIDENCE THAT THE BIOLOGICAL ACTIVITIES ARE CHARACTERISTICS OF THE PROTEIN. It is well known that various investigators, notably Dudley, Kamm and his collaborators, and later, Stehle, du Vigneaud and their colleagues, have been able partly or nearly completely to separate the oxytocic and vasopressor activities from each other. Therefore it was of great importance to demonstrate as convincingly as possible whether the two principal activities of posterior lobe extract are or are not part of the pure protein isolated according to the method we have described. Later we shall discuss the reconciliation of our findings with those of workers who isolated the two activities as separate substances of much higher potency.

Within the limitations of careful biological assay, different preparations of the pure protein are identical in either oxytocic or vasopressor activity in which one U.S.P. unit is represented by about 10 micrograms of protein nitrogen or 61 micrograms of protein. This statement is based upon work with five different preparations of the protein made from as many different batches of starting material. Such findings support the belief that oxytocic and vasopressor (including diuresis-inhibiting) activities are part of the protein and are present in ratios resembling those found in cruder extracts of posterior lobes of oxen. To furnish further evidence there will now be described efforts to detect oxytocic or vasopressor activities more potent than those of the pure protein. For this purpose fractions were secured (a) after electrophoretic migration of the protein, (b) after solubility tests, and (c) after ultracentrifugation.

Fractions secured after electrophoretic migration of the protein. After a dissolved protein has been electrolyzed in the Tiselius apparatus migration will occur slowly or rapidly depending mainly upon how close the pH of the solvent is to the isoelectric point. If migration is rapid or if electrolysis is continued long enough, fractions of the solution can be withdrawn from the limbs of the cells containing either virtually no protein or pure protein. In addition, as shown in figure 4, there appears to be a second component at pH 3.4-3.5 which could be separated from the main component and assayed. (At other pH values the same preparations appeared as one component which, however, was electrophoretically inhomogeneous.) The assays of all fractions were undertaken after nitrogen determinations had been made. The concentration of nitrogen in the "protein free" fractions was usually so low (8-20 micrograms per ml.) that the error of determination was relatively high and, in view of the lack of any change in potency, necessitated assay by only very sensitive methods such as the depressor effect on fowl blood-pressure.

Our results can be summarized by stating that after electrolysis of solutions of the protein at pH 3.4-3.5, the protein and "protein-free" fractions were indis-

tinguishable in activity in terms of nitrogen. The minor component appearing at pH 3.4-3.5 likewise did not differ significantly in potency when compared with the main component—a fact suggesting that it is derived from or closely related to the main component.

Fractions secured in solubility tests. In the experiment of figure 7, protein to various concentrations was dissolved in 0.5 M acetate buffer, pH = 3.90, and the equivalent of 6.5 grams of NaCl per 100 ml. was added. After the NaCl had been dissolved, only the first three mixtures (0.048-0.072 mgm. N per ml.) were clear solutions; the protein in excess of saturation was in suspension in the

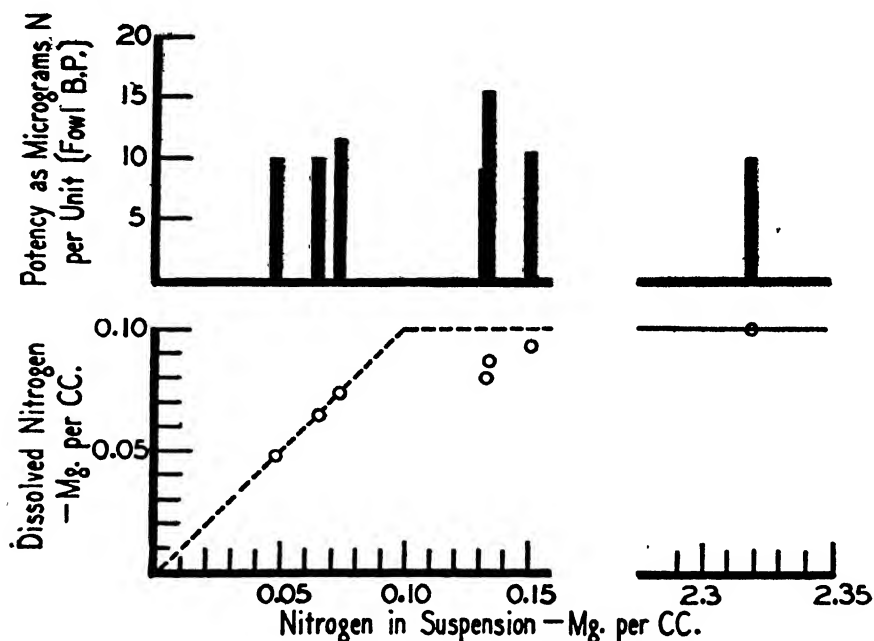


FIG. 7. THE OXYTOCIC ACTIVITY OF DISSOLVED PROTEIN IN RELATION TO AMOUNT DISSOLVED OR SUSPENDED IN 0.5 M ACETATE BUFFER, pH = 3.90, TO WHICH 6.5 GRAMS NaCl PER 100 ML. WERE ADDED

remaining solutions. The oxytocic activity (depressor effect on fowl blood-pressure) was determined in the supernatant fluids (dissolved protein). With the exception of one aberrant result (at about 0.135 mgm. of suspended protein nitrogen) which was not confirmed on immediate repetition, there were no significant variations in the activity of the supernatants. Had a less soluble potent impurity been present, the activity of the supernatant would have been lower when there was a great excess of suspended protein. If a more soluble potent impurity had been a contaminant, the activity would have been lower when all the protein was dissolved or when that present was only moderately in excess of saturation.

The legend of figure 8 describes the conditions under which that experiment

was performed. If a less soluble active impurity (hormone) had been present, the washed precipitated protein, *P*, would have been the more potent, and the supernatant, containing dissolved protein as *S*, would have been the less potent. If a more soluble active impurity (hormone) had been present, *P* would have been the less potent and *S* would have been the more potent. The amount of material available permitted two careful assays of *P* and *S* by both techniques. The mean results which are plotted show that the activities of *P* and *S* did not

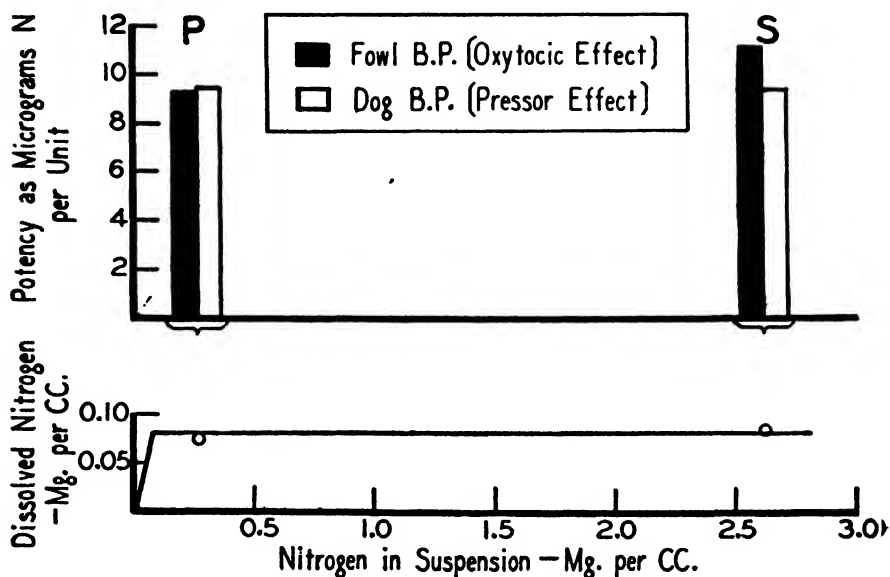


FIG. 8. The lower curve represents the solubility of pars neuralis protein. At the first point (0.266 mgm. N in suspension), the protein used had been washed three times by dissolving in 0.5 *M* acetate buffer (pH 3.95) and precipitating by the addition of NaCl (6.5 grams per 100 cc. of solvent). In this way, 54 per cent of the starting material had been removed. The oxytotic and pressor assays of this protein as the insoluble portion at the point indicated are given under *P* (upper rectangles). Assay of the supernatant (dissolved protein) was performed at *S* from a suspension (made by dissolving the protein in 0.5 *M* acetate buffer and adding 6.5 grams NaCl per 100 cc. of solvent) ten times as concentrated as at *P* and thirty times the concentration of protein in saturated solution in the NaCl acetate solvent.

The pharmacological activities of *P* and *S* whether tested for oxytotic or pressor effects do not differ significantly.

differ significantly in respect of either oxytotic or pressor effects. Therefore this experiment likewise supports the beliefs that oxytotic and pressor activities in a ratio similar to that of U.S.P. reference standard are parts of the pure protein and that principles more active than the protein could not be detected.

Fractions secured after ultracentrifugation of a solution of the protein. In experiments of this type the ultracentrifugation cell had a capacity of only 0.40 cc. Therefore assays were based, not upon the nitrogen of a given fraction of the column of liquid, but upon the distribution and total number of units placed in the cell before ultracentrifugation. Control assays of the same protein solution

not subjected to ultracentrifugation were performed for all the comparisons. In all cases solutions of the pure protein of the usual potency (10 micrograms $N \approx 1$ U.S.P. reference standard unit) were employed.

In the experiment of figure 9, the protein was found to be pure and was centrifuged until nearly all appeared to be contained in the lower half of the cell. Assay of the three fractions of the column showed that very little of the total activity (about 5 per cent) could be found in the upper half of the cell. About 6 per cent of the theoretical total activity could not be accounted for. It appears

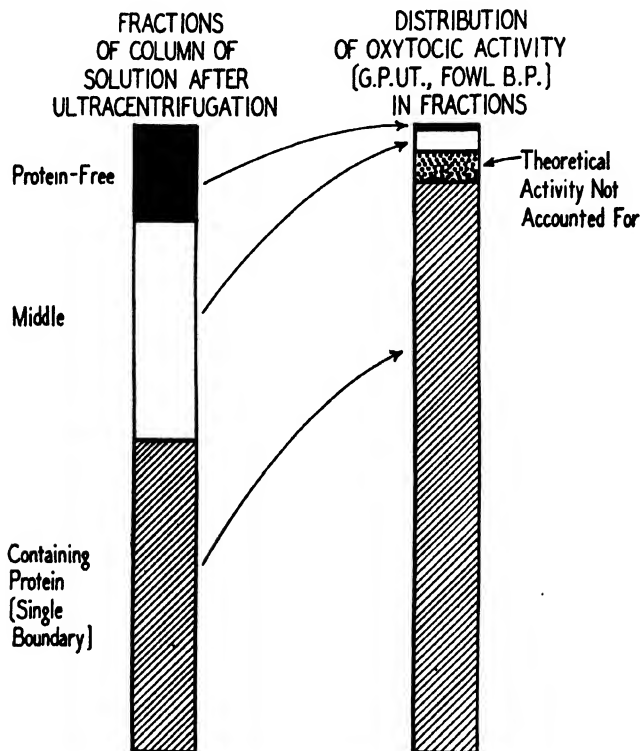


FIG. 9. ACTIVITY OF COLUMNS OF LIQUID IN CELL AFTER ULTRACENTRIFUGATION OF PROTEIN AS 1.05 PER CENT SOLUTION IN 0.1 M ACETATE BUFFER, pH = 3.30

fair to conclude that backward diffusion accounts for the small amount of active material in the protein-free portions of the column of liquid and that activity is associated with the protein.

It will be noted that assays for pressor activity were not performed in the experiment of figure 9. In the separation experiment of figure 10 (see also table 1 and fig. 6), both oxytocic and pressor activities were assayed in the liquid of the two parts of a separation cell^a after the protein, sedimenting as a single

^a The sieve plate supporting a piece of hardened filter paper was located at about a third of the height of the cell from the bottom. The total volume of the cell was 0.9 ml.

component, had all been collected in the lowest third of the cell. The slight activity found in the upper two thirds of the cell corresponded to the amount of protein left as estimated optically. A complete separation was impossible since diffusion, owing to the low molecular weight of the protein, caused an appreciable spreading of the boundary. Rectangles I, II, and III show how oxytocic and pressor activities were distributed in the protein-free and protein-containing parts of the cell. The theoretical total activity was computed from assays performed simultaneously on the same protein solution which had not undergone ultracentrifugation. It is clear that practically all the activity, whether oxytocic or pressor, is associated with the pure protein and that again there is no evidence favoring the presence of additional small non-protein components with high biological activity.

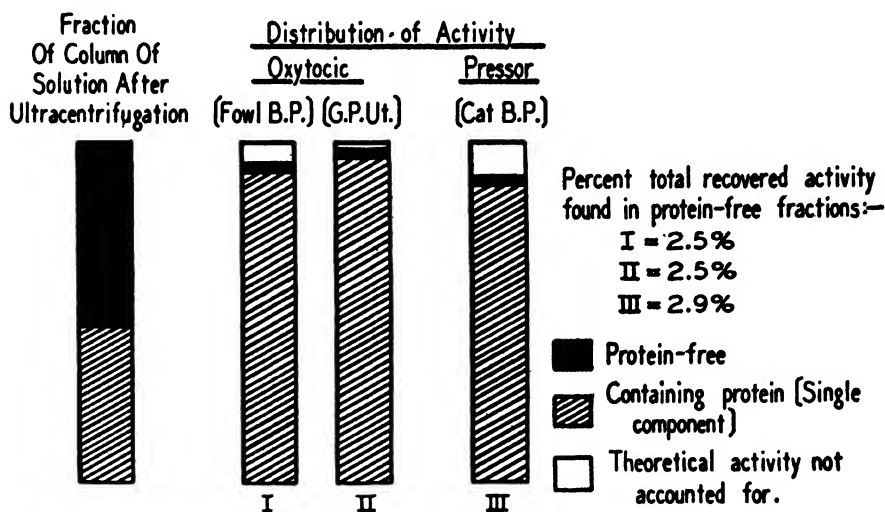


FIG. 10. Experiment similar to fig. 9. See also fig. 6. Ultracentrifugation of 1.10 per cent solution of protein in 0.17 *M* NaCl containing 0.05 *M* acetate buffer, pH = 3.35.

MISCELLANEOUS OBSERVATIONS. *Digestion by proteolytic enzymes.* A solution of the protein at pH 3 was digested by crystalline pepsin in an incubator (37°) for six days so that 36 per cent of the protein present had been digested as determined by the amount of non-protein N appearing. The incubated control had the same activity as the control kept in the refrigerator; the digested protein had about 75 per cent of the activity of the controls. Digestion by crystalline trypsin (70 per cent of protein digested) or crystalline chymotrypsin (95 per cent of protein digested) at pH 7.6 for 43 hours caused virtually complete destruction of activity compared with the control solution incubated at the same pH. In all these experiments only oxytocic activity was tested. The results are about what would be expected in view of earlier work on posterior lobe extracts.

The effect of reduction of —S—S— groups. Since in our protein nearly all the sulfur appears to be in the form of cystine, we made a preliminary investiga-

tion of the activity after reduction of the protein by thioglycollic acid or cysteine. Presumably dithio groups of cystine would thus be reduced to sulfhydryl groups.

In a typical experiment, 2.0 ml. of posterior lobe protein (0.364 mgm. N per ml.) were mixed with an equal volume of saline or of neutralized thioglycollic acid and 0.4 ml. of 0.1 N NaOH (final pH ca 7.5). Equal volumes of saline and thioglycollate were mixed to serve as another control. The protein solution not containing thioglycollic acid could be assayed only after it had been greatly diluted. The control solution containing only thioglycollic acid as well as the protein solution which had undergone reduction were injected in equally large doses of diluted mixture into the dog or fowl used for assay. Reduction of the protein by thioglycollate under the described conditions abolished more than 99.5 per cent of the activity when reduction had been allowed to proceed no longer than 2-5 minutes (5 experiments). Both vasopressor and oxytocic activities were affected to an equal extent. The alkalinized solutions of protein (non-reduced controls) appeared to be as active as saline solutions of the protein. The thioglycollic acid control solutions produced no pharmacological effect.

When similar experiments were performed with cysteine, the amino acid had a less pronounced effect probably because it is a less powerful reducing agent than thioglycollate. The pH of the solutions, whether controls or containing 30 mgm. of cysteine HCl for 0.182 mgm. of protein N, was raised to about 7.5. Cysteine Na solutions had no effect in doses equalling the maximum used for other solutions. After 2 minutes' reduction, the maximum loss of pressor activity was 44 per cent (2 experiments). After 5 minutes' reduction (2 experiments) 76 and 85 per cent of the oxytocic activity had disappeared. (The difference in time of reduction was arbitrarily chosen and has no significance.)

The action of the protein on the concentration of blood sugar. Doses equivalent to 1 and 3 U.S.P. units per kilo injected subcutaneously into fasted rabbits had no effect on the level of blood sugar estimated by the method of Shaffer and Hartmann as modified by Somogyi (22). Duplicate samples of blood were withdrawn before injection as well as at 1, 1.5, 2, and 3 hours after injection. Five units of the protein per kilogram body weight caused no change in the blood-sugar level of another rabbit. The subcutaneous administration of 2 units per kilogram of commercial posterior pituitary extract caused a moderate elevation of the blood glucose (77 mgm. per cent before injection; maximum rise to 119 mgm. per cent 1.5 hours after injection). We concluded that the protein probably does not affect carbohydrate metabolism so far as this can be judged by acute changes in the level of blood sugar.

DISCUSSION. The protein which we extracted from freshly frozen posterior lobes of oxen appears to contain, in constant amounts, multiple biological activities as shown by its ability to cause uterine contraction, a depressor effect in the fowl, a pressor effect in the cat and dog, and inhibition of diuresis in the rat.⁴ These activities are present in about the same ratios as in expressed

⁴ MacArthur (23) has not reported *in extenso* on the nature of the substance with multiple activities which he isolated.

tissue fluid or simple acid extracts of posterior lobes of oxen. Our results therefore harmonize well with Rosenfeld's study (7) of the ultracentrifugal sedimentation of oxytocic and pressor activity. This author suggested that the two activities can be separated by cleavage from a parent molecule and then exist as much smaller molecules sedimenting at a much slower rate. However if the protein-containing extract is kept as native as possible by avoiding heating in acid solution or some other drastic procedure, then the two activities sediment as if part of one (or more) protein molecules. The late Professor Abel long championed a similar view which he maintained in his last publication in this field (2). However, as Dale emphasized, the presence of large amounts of the melanosome-dispersing ("melanophore") hormone in his preparations is a valid argument against his position since this hormone is derived not from the *pars neuralis* but from the *pars intermedia*. The protein described in this report contains virtually no intermedin.

Many investigators have commented on the constancy of the ratio of oxytocic and vasopressor activities of beef posterior lobes and this fact suggests that these activities may be part of one molecule. However, in the whale it has been reported (24, 25) that oxytocic activity may be 8–10 per cent (sperm whale), 30 per cent (blue whale), or 40 per cent (finback) of the vasopressor activity in terms of U.S.P. reference standard. Although such findings can obviously be interpreted in different ways until more data have been gathered, they speak against rather than in favor of the belief that both activities are part of one molecule.

As a result of the pioneer work of Dudley (3) and especially of Kamm and his colleagues (4) followed by the confirmatory experiments of Stehle, du Vigneaud and their collaborators there can be no doubt that oxytocic and vasopressor principles can be separated from each other in highly potent form. The most potent oxytocic preparation reported contained 1 unit in 2 micrograms of solids (30 times the potency of the protein reported here); a vasopressor preparation containing 1 unit in 5 micrograms of solids (12 times as potent as our protein) has been described. There appear to be two possible explanations permitting reconciliation of these facts with our belief that a protein with multiple activities also can be isolated: (1) the protein isolated, although pure to the extent that present physico-chemical methods permit such a conclusion, is pharmacologically active because of the adsorption of the highly active separated principles, or (2) the protein, in part composed of active principles which can be separated from it, is elaborated by and stored in the *pars neuralis*.

It is well known that differential adsorption of the vasopressor principle by bentonite or artificial zeolites occurs easily under proper conditions (26, 27). The fact that this can be effected on an artificial zeolite if a posterior lobe extract in which cleavage of protein has been avoided be used (27), does not demand the conclusion that vasopressor activity is only in loose physical union with the proteins of the gland. A powerful adsorbent can be considered to affect drastically molecules as fragile as protein molecules. Oxytocic activity can be adsorbed by Fuller's earth, norite, talcum and PbS. Also, apparently pure crys-

talline hormone with both activities present, has been isolated on more than one occasion only to lose its biological activity with repeated recrystallization (28). In these instances we appear to be dealing with adsorption of the active principles and it cannot be denied that the biological activity of the protein here described may depend upon adsorbed oxytocic and vasopressor principles rather than upon chemical union (e.g. peptide linkages) of these principles with the protein. However, all the evidence we have obtained—constancy of activity in different preparations, absence of potency greater than that of the protein in fractions secured after electrophoresis, ultracentrifugation, or solubility tests, association of activity with the protein,—supports the view that the activities are part of the protein.

Moreover, the following experimental facts are explained only with great difficulty by the adsorption theory. It was found⁵ that the protein carrying the oxytocic-pressor activity could be spread as a surface film in an unfolded condition, since the thickness of the film was only 8\AA as measured by the optical method of Blodgett and Langmuir (29). If the activities had consisted of small units adsorbed to the protein, either the adsorbed active units would have gone into solution when the unfolding occurred or they would have continued to be associated with the denatured protein of the film. In the first case the underlying fluid should have contained all the activities and in the second the activities should have been recovered in the film, since it was not to be expected that unfolding of the hypothetical adsorbing protein should affect the potency of the assumed small units. The film was found to be nearly completely inactive and the potency of the underlying fluid in terms of protein N was less than that of the original material similarly diluted and concentrated for purposes of control. (The activity present could be accounted for by the amount of protein going into solution at the time of spreading.) It can be concluded that unfolding virtually destroys the activities indicating that some part or parts of the original configuration must remain intact if potency is to be preserved.

If the protein were subjected to heat perhaps at pH 3.5–4.0 following which the dialyzability of the two (or more) principles were to be investigated, useful information might be secured from following the ratio of activities dialyzed or retained (28). In our opinion, this information can be obtained only from careful serial experiments in which the rate of change is followed. We hope later to perform such experiments.

The second possible reconciling explanation is that a protein containing all the activities is formed in the *pars neuralis* but that drastic methods of extraction, autolysis, etc. facilitate or cause the liberation or cleavage of the active principles. An important and inescapable question is: If only a single protein with all activities is elaborated in the *pars neuralis*, is it secreted unchanged? Teleologically at least this appears to be improbable and should not be accepted without proof (28). Possibly specific enzymes liberate one or the other active fragments of the parent molecule depending upon specific demands of the

⁵ Cold Spring Harbor Symposia, 9: 272, 1941.

organism. So far as we are aware there have been no careful quantitative assays in search of multiple activities in single specimens of urine or blood under conditions leading to the expectation that at least one activity (e.g., that inhibiting diuresis) would be secreted in increased amounts. Such a problem is exceptionally difficult to solve and would demand elaborate preliminary control experiments.

We have already referred to the fact that the dialysate of crude extracts obtained from fractionating extracts of fresh glands contains a considerable amount of biologically active material. It is our belief that this is probably the oxytocic and vasopressor principles, whatever their origin, mixed with other non-protein nitrogenous substances. Large quantities of perfectly fresh posterior lobes of oxen cannot ordinarily be obtained but must be collected, frozen and later dissected. If the protein is the sole source of active principles possibly it undergoes *post mortem* enzymatic cleavage sufficiently to account for any coexisting dialyzable principles which are presumed to be separate.

The cystine content of the protein we isolated is extraordinarily high (16.1 per cent or 4.3 per cent cystine sulfur). It appears that all the sulfur is present as this amino acid. Other investigators have reported the sulfur content of purified oxytocic or vasopressor principles as ranging from 3.1–4.5 per cent. Improved methods of determining cystine-cysteine and methionine have become available since these reports were made and the figures given by earlier authors as representing cystine cannot justifiably be compared with ours. However, it appeared that about 10–12 per cent of highly purified oxytocic or vasopressor principle was represented by cystine. It is noteworthy that in purifying the vasopressor principle Irving, Dyer and du Vigneaud (30) early separated an impurity containing a large amount of cystine. Although Sealock and du Vigneaud (31) found that whether the sulfur was present in —S—S— or —SH form (reduced by cysteine) seemed not to affect either vasopressor or oxytocic activity, Gulland and Randall (32) as well as Freudenberg and his collaborators (33) concluded that reduction definitely impairs activity at least of the oxytocic principle. Freudenberg and others found that nearly complete inactivation followed treatment of purified oxytocin with neutral or weakly alkaline sulfite or with hydrogen in the presence of Pd; sulfite as well as other means of reduction were employed by Gulland and Randall. In our own experiments, reduction by cysteine lowered both vasopressor and oxytocic (fowl depressor) potency of solutions of the protein. Reduction by thioglycolic acid caused nearly complete inactivation. Therefore we believe that the protein becomes inactive if the dithio groups (cystine) are completely reduced to sulphydryl groups.

SUMMARY

The isolation of a protein from frozen posterior lobes of oxen is described. It appears to be pure so far as this can be demonstrated by constant solubility and by "schlieren" patterns in the ultracentrifuge. Although some inhomogeneity was found in the Tiselius electrophoresis apparatus, reasons are given for believing this to be derived from a pure protein. The molecular weight is

about 30,000. The isoelectric point is about pH 4.8. Empirical analysis revealed an unusually high percentage of S (4.9 per cent) which is almost entirely present as part of cystine.

Oxytocic, vasopressor, and diuresis-inhibiting activities are all present in ratios resembling those of U.S.P. reference standard. Ten micrograms of nitrogen or 61 micrograms of solids are approximately equivalent to 1 U.S.P. unit. Experiments designed to demonstrate that the oxytocic and vasopressor activities are moieties chemically united with the protein are described.

Reduction of the cystine in the protein by thioglycolic acid nearly abolishes the activity. In this respect cysteine is much less effective.

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AN ELECTROPHORETIC STUDY OF MIXTURES OF OVALBUMIN AND YEAST NUCLEIC ACID

By LEWIS G. LONGSWORTH AND D. A. MACINNES

(From the Laboratories of The Rockefeller Institute for Medical Research)

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INTRODUCTION

In an ideal electrophoresis of a solution of two components the volumes swept through by the rising and descending boundaries due to each component are identical and are proportional to the mobilities of the separate components. Moreover, the area under each "peak" in the electrophoretic pattern is proportional to the stoichiometric concentration of that component in the mixture to which it is due. In this ideal or limiting case the patterns for the two sides of the channel of the Tiselius electrophoresis cell are mirror images of each other.

The two patterns are, however, never exact mirror images. The authors have already discussed¹ the asymmetries, including the δ and ϵ effects, that are observed in the electrophoretic patterns of a single component and deviations of this type are, of course, to be expected in the electrolysis of mixtures. In the case, however, of some mixtures asymmetries were observed² which appeared to be due to interaction between the constituents. This led to the research, to be described below, in which the effect of interaction of components on electrophoretic patterns has been investigated further, using mixtures of ovalbumin and yeast nucleic acid. It is also the purpose of this paper to indicate a method for the electrophoretic analysis of mixtures in which certain types of interaction occur, and to discuss the manner in which the asymmetries in the electrophoretic patterns arise.

EXPERIMENTAL

The ovalbumin used in this research was prepared by the method of La Rosa³ and was recrystallized three times. The nucleic acid was a sample prepared under the direction of Dr. P. A. Levene of these Laboratories. This material was electrophoretically homogeneous, *i.e.* gave a single sharp peak,

¹ Longworth, L. G., and MacInnes, D. A., *J. Am. Chem. Soc.*, 1940, **62**, 705.

² Longworth, L. G., Cannan, R. K., and MacInnes, D. A., *J. Am. Chem. Soc.*, 1940, **62**, 2580.

³ La Rosa, W., *Chemist-Analyst*, 1927, **16**, 3.

other than the δ and ϵ effects, in the electrophoretic pattern. It was observed, however, that some of the acid was lost on dialysis in cellophane tubing. Hence it was necessary to dialyze for a definite interval and correct for the material lost.

The patterns of Fig. 1 were obtained in the electrophoresis of a mixture of 1.15 per cent ovalbumin, P , and 0.50 per cent nucleic acid, N , in a 0.1 N sodium acetate buffer at pH 5.34. In this buffer both components carry appreciable negative charges, the mobilities having the values $u_P = -2.8 \times 10^{-5}$ and $u_N = -13.1 \times 10^{-5}$. Under these conditions a pattern for the mixture is essentially the sum of the patterns for the two components which were obtained separately.

The patterns of Fig. 2 were obtained with a similar mixture, 1.15 per cent P and 0.67 per cent N , but in a 0.1 N sodium acetate buffer at pH 4.63. In this solvent the protein still has a small negative mobility, -0.2×10^{-5} , but the patterns exhibit asymmetries⁴ that cannot be explained in terms of the δ and ϵ effects alone. Thus the displacements of the boundaries b and γ , Fig. 2, are proportional to the normal mobilities u_P and u_N , respectively, but the displacement of the boundary β corresponds to a mobility some eight times greater than the mobility of ovalbumin at this pH, while the displacement of the boundary c corresponds to a mobility appreciably less than that of nucleic acid. Moreover, the area under the boundary at γ corresponds, after correction for the dilution due to the δ effect,¹ to only 0.56 per cent of nucleic acid whereas the actual concentration of that component was 0.67 per cent. Also the area under the boundary at c would correspond to 0.93 per cent if this were due entirely to nucleic acid. The results of this experiment are, as will be shown below, consistent with the assumption that the boundaries γ and b are due to nucleic acid and ovalbumin, respectively, moving with their normal mobilities, but whose concentrations have been modified by interaction. The boundaries β and c will, on the other hand, be shown to arise from the complexes due to combination of the components. It will be shown that the displacement of these boundaries involves the equilibrium between the components and the complex in addition to the motion of these substances in the existing electric field.

Although the patterns are not reproduced in this paper, experiments have been performed in which each of the four variables, *i.e.* pH, ionic strength,

⁴ We have observed similar asymmetries in the patterns of mixtures of ovalbumin and salmine, which is a basic protamine, except that in this case the patterns approach those of a normal mixture at pH values *below* the isoelectric point of the protein.

⁵ It may be noted in Fig. 2 that the gradients of refractive index in the boundary at c are not symmetrical about the ordinate passing through the maximum gradient. In measuring the displacement of this boundary it is necessary, therefore, to follow the procedure suggested by one of us (Longworth, L. G., *Ann. N. Y. Acad. Sc.*, 1941, **41**, 267).

protein concentration, and nucleic acid concentration, have been altered systematically. The results, some of which are given in Table I, indicate that

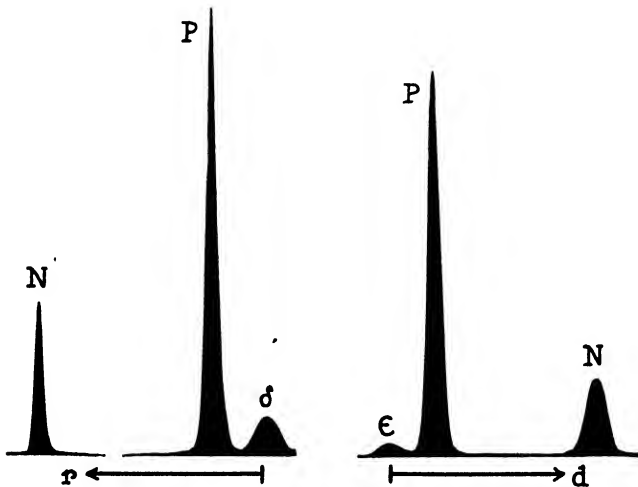


FIG. 1

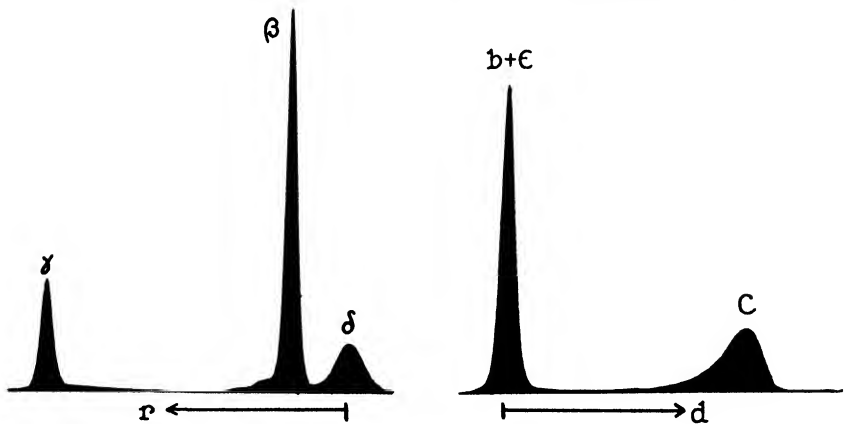


FIG. 2

FIG. 1. Electrophoretic patterns of a mixture of ovalbumin, 1.15 per cent, and yeast nucleic acid, 0.50 per cent, in a 0.1 N sodium acetate buffer at pH 5.34. The patterns were obtained after electrophoresis for 5000 seconds at 7.22 volts per cm.

FIG. 2. Electrophoretic patterns of a mixture of ovalbumin, 1.15 per cent, and yeast nucleic acid, 0.67 per cent, in a 0.1 N sodium acetate buffer at pH 4.63. The patterns were obtained after electrophoresis for 7000 seconds at 6.97 volts per cm.

pattern asymmetries of the type described above are enhanced by decreasing the ionic strength or by increasing the concentrations of the protein or the nucleic acid or both. The patterns of Figs. 1 and 2 illustrate the important

rôle that pH plays in such experiments. Experiments at pH values below 4.6, the isoelectric point of ovalbumin, are complicated by the partial precipitation of the components. For electrophoretic study, removal of the precipitate that forms on dialysis of the mixture⁶ is not sufficient since additional precipitation frequently occurs, during an experiment, that interferes with observation of the boundaries. Thus in an experiment at pH 4.45, with conditions otherwise similar to those represented by Fig. 2, only a slight precipitate formed on dialysis and this was removed before electrolysis but during the latter procedure an optically opaque stratum of precipitate formed in the channel between the δ and the β boundaries on the anode side. This precipitation was probably due to the decreased ionic strength in this region of the cell arising from the δ effect and suggests that the solubility of the ovalbumin-nucleic acid complex is very sensitive to the ionic strength as well as to the pH.⁷

Electrophoretic Analysis of Ovalbumin-Nucleic Acid Mixtures

The distribution of the components in the cell before and after electrophoresis is shown in Figs. 3 and 4. The boundaries between the buffer solution and solution (in the buffer) of ovalbumin at an initial or total concentration p_i and nucleic acid at a total concentration n_i were present in the planes a and α of Fig. 3 at the time the potential was applied. After passage of a current the boundaries in the anode side of the channel (Fig. 4) had swept through the volumes V_γ and V_β , while on the cathode sides the volumes were V_b and V_c .

The material recovered from the channel between the boundaries at β and γ was found by direct experiment to be pure nucleic acid at a "separated" concentration n_s whereas that between the boundaries at b and c was pure ovalbumin at a corresponding separated concentration p_s . Since the passage of an electric current does not produce changes of composition in the body of a

⁶ The precipitate formed in a 15 ml. sample of a 1.15 per cent *P*-0.58 per cent *N* mixture in a 0.1 *N* sodium acetate buffer at pH 4.34 was separated, dissolved in 15 ml. of a 0.1 μ sodium phosphate buffer at pH 6.80, dialyzed against this buffer, and analyzed electrophoretically. The pattern indicated no interaction at this pH and corresponded to a mixture of 0.40 per cent *P* and 0.13 per cent *N*. Moreover the ovalbumin thus separated electrophoretically from the nucleic acid was still native and showed the same relative amounts of the A_1 and A_2 modifications (*cf.* footnote 2) as in the original stock solution of the protein. It thus appears that precipitation under the conditions outlined here does not denature the protein and that the complex with nucleic acid is reversibly dissociated at pH values sufficiently above the isoelectric point of the ovalbumin.

⁷ Our observations concerning the influence of pH and ionic strength upon the solubility of the ovalbumin-yeast nucleic acid complex are in qualitative accord with those of Hammarsten and Hammarsten (Hammarsten, E., and Hammarsten, G., *Acta med. Scand.*, 1928, **68**, 199) on the complex formed by the related material, thymus nucleic acid, and ovalbumin.

homogeneous solution the protein solution in the bottom section of the channel, and the buffer solution in the two sides of the top section, remain unchanged. Moreover, ovalbumin and nucleic acid neither enter nor leave the cell.⁸ Consequently if the pattern for one side of the channel indicates a loss by electrophoretic migration of one of these components the pattern for the other side should indicate a corresponding gain of that component.

Referring to Fig. 4, the quantity of protein initially present in the volume V_c was $V_c p_t$ while that present in the same volume after electrolysis was $(V_c - V_b) p_s$. Thus the loss of this component from the cathode side was

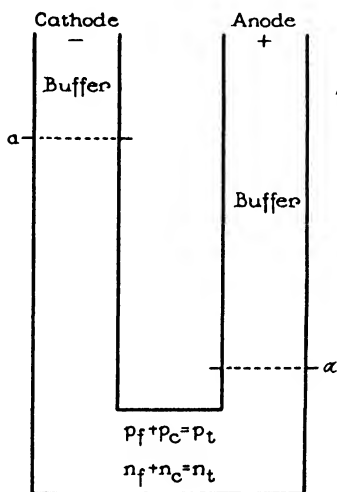


FIG. 3

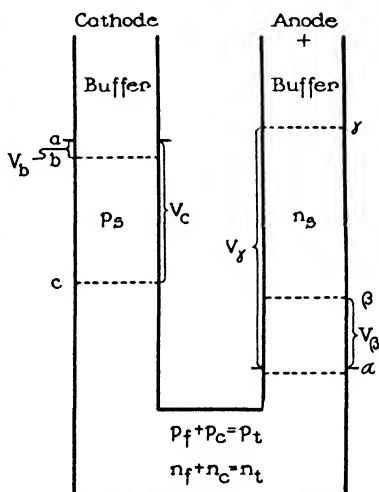


FIG. 4

FIG. 3. Diagrammatic representation of the initial distribution of materials in the electrophoresis channel.

FIG. 4. Distribution of materials in the channel after electrophoresis.

$V_c p_t - (V_c - V_b) p_s$ and this should equal the gain on the anode side, namely, $V_\beta p_i \rho_P$ in which ρ_P is the dilution factor of the protein at the δ boundary. Therefore,

$$V_c p_t - (V_c - V_b) p_s = V_\beta p_i \rho_P \quad (1)$$

and similarly for the nucleic acid,

$$V_c n_t = V_\beta n_i \rho_N + (V_\gamma - V_\beta) n_s \quad (2)$$

⁸ The buffer electrolytes, on the other hand, move through the cell but the quantity of these materials present at any instant remains constant, except for negligibly small effects due to volume changes accompanying electrophoretic separation of the components if the partial volumes of the latter are not additive.

in which ρ_N is the corresponding dilution factor for the nucleic acid. All of the terms in equation 1, for example, can be obtained from the electrophoretic patterns of the rising and descending boundaries, with the exception of p_i , if the specific refractive increment of the protein is known. Thus from the two patterns the total concentration of protein, p_i , may be computed whether interaction takes place or not. This is obviously also true of equation 2, and n_i . Since the composition of the initial solution, and therefore p_i and n_i , were known a comparison of the observed and computed concentrations affords a test of our interpretation of the phenomena occurring during electrophoresis.

In making computations with the aid of equations 1 and 2 it will be assumed that the dilution factors are given by the relation

$$\rho_P = \rho_N = (A_i - A_\delta)/A_i \quad (3)$$

in which A_i is the total area of the electrophoretic pattern and A_δ that due to the δ boundary. The assumption that $\rho_P = \rho_N$ is in accord with the theory of Henry and Brittain⁹ who showed, for a somewhat simpler system, that at the δ boundary "the advancing column will hold its constituent ions in the same relative proportion as in the original sol." The additional assumption contained in equation 3; namely, that $\rho = (A_i - A_\delta)/A_i$, represents an approximation based upon the observation that the gradients of buffer salts in the δ boundary, being similar to those in the ϵ boundary, are small in comparison with the gradients of P and N .

Equation 1 may be rearranged to give

$$p_i = \frac{V_c - V_b}{V_c - V_{\beta\rho}} p_b = \frac{V_c - V_b}{V_c - V_{\beta\rho}} k_P A_b \quad (1')$$

in which A_b is the area under the boundary at b and k_P is a factor converting this area into protein concentration. This factor, whose value is 0.003776, depends only upon constants of the apparatus and the specific refractive increment of ovalbumin, taken as 0.00184 for the Hg blue and violet lines used in the present research. Similarly, equation 2 may be rearranged to

$$n_i = \frac{V_\gamma - V_\beta}{V_c - V_{\beta\rho}} n_b = \frac{V_\gamma - V_\beta}{V_c - V_{\beta\rho}} k_N A_\gamma \quad (2')$$

in which $k_N = 0.005185$, the specific refractive increment for nucleic acid being taken as 0.0013.¹⁰

⁹ Henry, D. C., and Brittain, J., *Tr. Faraday Soc.*, 1933, **29**, 798.

¹⁰ This value is taken from the work of Seibert and Watson (Seibert, F. B., and Watson, D. W., *J. Biol. Chem.*, 1941, **140**, 55). It may be noted in this connection that the conclusions of our paper are actually independent of the values of the specific refractive increments since the concentrations of our stock solutions of both ovalbumin and nucleic acid were determined refractometrically.

The data necessary for computations with the aid of the foregoing relations, as obtained in five experiments in which the relative proportions of ovalbumin and nucleic acid were varied, are given in Table I. The data obtained from the patterns of Fig. 2 are given in the fourth column of this table. The other patterns were qualitatively similar. The areas (lines 1 to 5) under the peaks of these patterns are in arbitrary planimeter units whereas the displacement volumes (lines 6 to 9) are in milliliters per second per unit potential gradient. Line 10 of Table I contains the concentration of ovalbumin used in each experiment whereas line 11 contains the value of the concentration of albumin computed with the aid of equation 1'. Lines 12 and 13 contain the corre-

TABLE I

Electrophoretic Analysis of Mixtures of Ovalbumin and Yeast Nucleic Acid in 0.1 N Sodium Acetate at pH 4.63 and 0°C.

Experiment No.	1	2	3	4	5
1. A_b	133.0	273.0	254.5	255.5	231.0
2. A_c	149.5	84.5	151.5	179.5	296.0
3. A_β	146.0	294.0	274.5	265.5	247.5
4. A_γ	96.0	33.5	68.0	89.5	154.0
5. A_δ	44.0	29.0	60.0	77.5	120.5
6. $V_b \times 10^5$	0.34	0.26	0.30	0.33	0.45
7. $V_c \times 10^5$	10.46	8.49	9.18	9.43	9.89
8. $V_\beta \times 10^5$	2.35	1.10	1.94	2.37	3.86
9. $V_\gamma \times 10^5$	13.02	12.36	12.76	12.94	13.47
10. Ovalbumin taken, <i>per cent.</i>	0.58	1.15	1.15	1.15	1.15
11. Ovalbumin computed, equation 1'	0.60	1.13	1.13	1.17	1.19
12. Nucleic acid taken, <i>per cent.</i>	0.67	0.25	0.52	0.67	1.16
13. Nucleic acid computed, equation 2'	0.63	0.26	0.51	0.66	1.11

sponding values for nucleic acid. The average difference between the observed and computed values is 2.4 per cent for the ovalbumin and 3.5 per cent for the nucleic acid. It is of interest that if interaction were neglected and the area A_δ interpreted as due entirely to ovalbumin the average difference between the observed and computed values of p_i would be 6.9 per cent whereas if the area A_c were interpreted as due entirely to nucleic acid the average difference between the observed and computed values of n_i would be 45 per cent.

The Interaction between Ovalbumin and Yeast Nucleic Acid

In considering the probable nature of the interaction between ovalbumin and nucleic acid it will be assumed that they combine reversibly to form a complex $X(= PN_r)$



in which the velocity constant for the forward reaction is k_1 and for the reverse reaction k_2 , and the equilibrium constant is $K = \frac{k_2}{k_1}$. The available evidence indicates that the complex has a mobility, u_x , intermediate between u_P and u_N . For the purpose of this discussion no restriction is placed on ν . It doubtless varies with both pH and ionic strength and may vary, as in the precipitin reaction,¹¹ with the concentrations of P and N . In the electrophoresis of such a mixture the equilibrium shown in equation 4 is not disturbed in the body of the solution by the migration of the constituents P , N , and X . At the boundaries, however, the tendency of the components to separate, due to their mobility differences, is accompanied by a disturbance of the equilibrium. The extent to which the equilibrium will shift to compensate for the altered conditions produced by electrophoretic separation depends upon the magnitudes of the velocity constants in comparison with the rate of separation. The following cases may be distinguished.

1. If k_1 is small and k_2 large, K will also be large. Under these conditions the complex is essentially completely dissociated and the mixture will behave as a normal mixture of P and N .

2. If k_1 is large and k_2 small then K will be small. This system behaves like a mixture of the complex and either P^{12} or N , depending upon which is in excess. If neither is in excess a single boundary, due to X alone, will be present in each side of the channel.

3. If k_1 and k_2 are both small and of similar magnitudes then K will be near unity. In this case finite concentrations of P , N , and X will exist at equilibrium. The mixture will behave like a normal mixture of the three components since the adjustment of the equilibrium is slow in comparison with the rate of electrophoretic separation.

4. If the rate of adjustment of the equilibrium is comparable with the rate of

¹¹ See, for instance, Kendall, F. E., *Ann. N. Y. Acad. Sc.*, in press.

¹² From the description by Stenhagen and Teorell (Stenhagen, E., and Teorell, T., *Tr. Faraday Soc.*, 1939, **35**, 743)-of their electrophoresis experiments on mixtures of serum albumin and thymus nucleic acid it appears possible that this system corresponds to case 2. Thus only two boundaries were present in each side of the channel and the patterns appeared to be symmetrical. Their analyses showed both nucleic acid and protein to be present in the region between the two boundaries on the anode side but essentially pure protein in the corresponding region on the cathode side, thus indicating protein to be present in excess.

Seibert (Seibert, F. B., *J. Biol. Chem.*, 1940, **133**, 593) has observed somewhat similar phenomena in her electrophoretic studies of the naturally occurring mixture of protein and nucleic acid from the tubercle bacillus. It is of considerable interest that her separation of these components, by salt precipitation, was much more effective at alkaline reactions, where both components are negatively charged, than at acid reactions.

electrophoretic separation, the behavior will be difficult to predict although one would expect the pattern to depend upon the rate of separation of the constituents.

5. If k_1 and k_2 are both large and of the same order of magnitude the equilibrium is adjusted as rapidly as required by the electrophoretic separation of the components. Consequently only two boundaries, aside from the δ and ϵ effects, appear in each side of the channel but the patterns are quite different from those of a normal mixture of two components.¹³ The patterns shown in Fig. 2 and those for which data are given in Table I appear to be compatible with the conditions postulated in this last case. It is of interest to visualize how these patterns may arise.

The initial conditions in the electrophoresis cell may be represented by Fig. 3 in which the concentrations of combined, free, and total protein in the mixture are p_c , p_f , and p_t , respectively, and the corresponding concentrations of nucleic acid are n_c , n_f , and n_t . The conditions in the cell after electrophoresis are indicated diagrammatically in Fig. 4 in which, as has been stated, pure N is present at a concentration n_s in the region between the two leading boundaries on the anode side and pure P at a concentration p_s in the region between the two boundaries on the cathode side. The sequence of events leading from the initial to the final state is as follows.

In the anode side of the channel the free N in the mixture escapes and moves upward with its normal mobility to a position γ . The complex X also moves upward through the plane α but in so doing tends to leave the slower moving P behind. Consequently a portion of the complex X dissociates in order to maintain the equilibrium indicated in equation 4. The N resulting from this dissociation moves ahead and fills the volume between the boundaries β and γ . The resulting concentration, n_s , of the separated nucleic acid is thus the sum of the concentration, ρn_f , due to the free acid in the body of the mixture and that arising from the decomposition of the complex. The factor ρ , it will be recalled, corrects for the dilution in the δ boundary. The protein P resulting from the partial dissociation of the complex, X , accumulates in the volume V_β

¹³ It may be noted that in all of the cases considered here, with the possible exception of the fourth case, a qualitative symmetry is retained by the patterns insofar as the same number of boundaries, exclusive of the δ and ϵ effects, is present in both channels. In some mixtures, however, even this type of symmetry is not observed. Thus Chargaff, Ziff, and Moore (Chargaff, E., Ziff, M., and Moore, D. H., *J. Biol. Chem.*, 1941, **139**, 383), in their electrophoretic studies of serum albumin-heparin mixtures, frequently observed three boundaries in one channel and two in the other. We have made similar observations with mixtures of ovomucoid and nucleic acid. Asymmetries of this type have not been satisfactorily explained but are, possibly, a reflection of the known complexity of whole serum albumin (McMeekin, T. L., *J. Am. Chem. Soc.*, 1940, **62**, 3393) on the one hand and ovomucoid on the other (*cf.* footnote 2).

at the concentration ρp_f . Due to the dissociation occurring in the boundary at β , the displacement, V_β , of this boundary is proportional to neither u_x nor u_P but has an intermediate value.

In the cathode side of the channel the nucleic acid, N , instead of escaping from the mixture, migrates into it. The complex thus tends to find itself in a region devoid of N and dissociates in the boundary at c , maintaining the equilibrium. The displacement, V_c , of this boundary is again proportional to neither u_N nor u_x but to an intermediate value. Pure P is left behind in the region between b and c at a concentration p_b which is greater than p_f but less than p_i . This material migrates with the normal mobility of P and hence the displacement V_b is proportional to u_P .

Much effort has been expended in an attempt to compute the mass action constant, K , of equation 4. For this computation from the electrophoretic data it is necessary to find a value of u_x ; i.e., the mobility of the complex. However, as indicated above, the motion of the boundaries involved also includes an effect due to the decomposition of this complex, making direct determination of this quantity impossible.

SUMMARY

Electrophoretic patterns of mixtures of ovalbumin and yeast nucleic acid indicate that the constituents migrate independently of each other in buffer solutions of 0.1 ionic strength and at pH values somewhat higher than the isoelectric point of the protein. In the isoelectric region, however, the patterns from the two sides of the channel exhibit asymmetries that can be explained by assuming the existence in the mixture of appreciable concentrations of a reversibly dissociable complex between the components. Formation of this complex is favored by increasing concentrations of the components and decreasing ionic strength. At pH values below the isoelectric point partial precipitation of the complex occurs.

The patterns obtained from each side of the channel in the electrophoresis of a mixture of two components, which form a dissociable complex, indicate only two boundaries, aside from the δ and ϵ effects. One of these is a normal boundary whose displacement is proportional to the mobility of a component that has separated from the mixture. In the other boundary, however, dissociation of the complex occurs and consequently the displacement of this boundary corresponds to the mobility of neither component nor to that of the complex. Moreover, the areas under the refractive index gradient curves are not proportional to the stoichiometric concentrations of the components. However, equations are developed with the aid of which an electrophoretic analysis of the mixture is possible. This analysis requires the use of data from the patterns of both channels.

THE MAGNETIC BEHAVIOR OF CATALASE

BY L. MICHAELIS AND S. GRANICK

(From the Laboratories of The Rockefeller Institute for Medical Research)

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Pauling and Coryell's (1) measurements of the magnetic properties of hemoglobin and several of its derivatives have revealed that observations along these lines are capable of contributing a great deal to the elucidation of the chemical structure of such compounds. Hemoglobin has turned out to be one of the strongest paramagnetic substances whereas oxyhemoglobin is not paramagnetic at all, indicating a profound change in the nature of the chemical bonds between iron and the attached groups as a result of the combination with molecular oxygen. Such a surprising result suggests extending this study to other heavy metal complexes of biological interest. It is the task of this paper to extend such measurements to catalase. Here, the task is very much more difficult than for hemoglobin. In addition to the difficulty of obtaining enough material, there is the risk of denaturation on thorough drying. It is not advisable to investigate the dried crystals of catalase. It is not feasible, either, to attempt measurements in solution since the solubility is too small within the permissible pH range. So one has to resort to investigating suspensions of the wet crystals. Fortunately, such a suspension in a suitable phosphate buffer is stable enough and sedimentation is negligible. However, a further difficulty remains, namely, that catalase has nearly four times the molecular weight of hemoglobin. Its iron content is 0.1 per cent approximately; *i.e.*, about one quarter that of hemoglobin. So it is impossible to obtain solutions or even suspensions comparable in iron content per cubic centimeter with that of readily obtainable concentrations of hemoglobin solutions.

Supposing the susceptibility per gram-atom Fe to be the same as in hemoglobin (in reality it is even smaller), a solution or suspension of catalase would produce only one quarter of the pull in a magnetic field of that of a hemoglobin solution of equal concentration. For this reason the apparatus used by Pauling and Coryell is scarcely sensitive enough. Recently a modification of the method has been developed in this laboratory which was originally used for the assay of free semiquinone radicals in solution. It is adequate also for the present task. This method is, like that of Pauling and Coryell, essentially Gouy's method converted into a differential method in order to obtain higher sensitivity. Some technical details have been published recently and since the method

is being still further refined, it may suffice to describe its principles roughly, postponing the ultimate details until some later time.

The solution to be measured is filled into the upper compartment of a cylindrical double vessel (Fig. 1), the lower compartment of which contains either water or some solution of suitable susceptibility which is never changed during a series of measurements. Only the contents of the upper compartment are

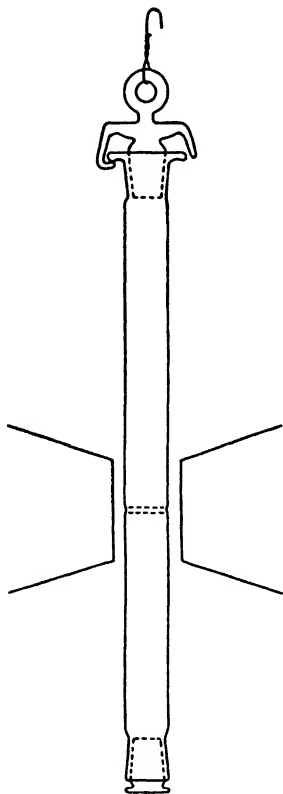


FIG. 1

varied. This vessel is suspended from one pan of a balance so that the diaphragm of the vessel is located between the centers of the pole pieces of an electromagnet. When the magnetizing current is switched on, the pull of the magnetic field on the upper and the lower compartment of the vessel is in opposite directions, and only the difference of pull is measured. This increases the sensitivity of the Gouy method very considerably. This differential method was first used, at least for solutions, by Freed and Kasper (2). The pull is measured in the following manner: The semi-micro, magnetically damped balance is equipped with a scale at the pointer with 200 divisions, each corresponding to

about 1/100th of a milligram, which is read through a microscope. What is observed is only the maximum deflection from the resting position brought about by abruptly switching on the current. Such readings are reproducible to ± 2 lines of deflection, and a series of ten readings gives a reliable average value. The current intensity is adapted to give a deflection of 20 to 120 lines. It was ascertained that deflections within all current intensities used, namely up to 10 amperes, are proportional to the square of the amperage. All readings are recalculated accordingly to 10 amperes.

The significance of each line of deflection, in terms of susceptibility, depends on the diameter of the vessel, the distance of the pole pieces, and the amperage. For each vessel, with given pole distance, the "vessel constant," *i.e.* the factor by which the number of lines of deflection must be multiplied to obtain susceptibility in c.g.s. units, is determined as follows. Keeping the contents of the lower, compensating compartment constant, the pull is measured first with air in the upper compartment, then with water in it. The difference corresponds to a change of susceptibility $= 0.74 \times 10^{-6}$, of which 0.2×10^{-6} is that of air and -0.72×10^{-6} that of water. Now, 0.74×10^{-6} divided by the difference of the numbers of lines of deflection, each recalculated for 10 amperes, for air and for water, is the vessel constant.

In an actual experiment, the upper compartment is filled with the solution, then with the buffer or solvent of the same composition, but not containing the dissolved (or suspended) substance to be investigated. The difference between the two values, expressed in lines of deflection, multiplied by the vessel constant, yields directly the increment of susceptibility due to the substance being measured. Small corrections may be necessary; however, they are quite irrelevant for the present purpose and may be omitted from this outline of the method. The increment in susceptibility divided by the grams of the specific substance in 1 cc. gives the susceptibility per gram of the substance. Multiplying this figure by 56 gives χ , the susceptibility of 1 g-atom of iron, in the form of catalase. Herefrom the magnetic moment, per gram atom of iron, μ , is obtained by the equation

$$\mu = 2.84 \sqrt{\chi \cdot T} \quad \text{Bohr magnetons}$$

where T is the absolute temperature at which the measurement has been made.

To check the method, the magnetic moment of $K_3Fe(CN)_6$ was determined in a 1/250 M solution, another in a 1/30 M solution, with the results: 2.44 resp. 2.25 Bohr magnetons using 1.7 cc. of the solution to fill the vessel. The first of these measurements is based on an amount of about 1.7 mg. of the substance, giving a differential deflection compared with pure water of about 25 lines. The acknowledged value is 2.33 magnetons; the agreement is satisfactory in spite of the minute amount of substance used. In one single experiment with a so-

lution of crystallized guinea pig ferro-hemoglobin in solution we found $\mu = 5.25$ per g-atom of hemoglobin-iron, as compared with 5.46 according to Coryell and Pauling.

The experiments with crystallized catalase were performed with a preparation made from beef liver according to the method of Sumner and Dounce (3), recrystallized once. The thin flat platelets were suspended in a mixture of equal volumes of Sørensen's phosphate buffer pH 7.4, and water. This buffer was suitable for keeping the crystals in suspension without any appreciable sedimentation occurring. The iron content of the dry catalase was 0.0918 per cent. The iron content of the final suspension per cc. was 0.0586; 0.0568; 0.0593; 0.0604; 0.0606; 0.0595; the average 0.0596 mg. This analysis was made colorimetrically with the *o*-phenanthroline complex (Hummel and Willard (4)) on samples containing 0.03 to 0.1 mg. Fe. The samples of the catalase suspension were digested with 1 cc. concentrated H_2SO_4 and 0.3 to 0.5 cc. HNO_3 and two drops of 70 per cent perchloric acid in a 100 cc. Kjeldahl flask. Blanks were determined correspondingly. The following remarks may be helpful. Prolonged heating after SO_3 fumes start to come off should be avoided to prevent as much as possible the formation of pyrophosphate and anhydrous ferric sulfate. After the digestion is complete, 10–20 cc. of water are added and the solution kept boiling slowly for an hour to decompose any pyrophosphate which would seriously interfere with the later development of the color. Even after this treatment, the full development of the color with phenanthroline takes some time. If the solution, before adding phenanthroline, is allowed to stand for 24 hours, the full color will be developed in several minutes. The final result, whether attained the one way or the other, is the same. The interference of pyrophosphate is serious if not well taken care of, but can be entirely overcome once the source of error is recognized.

The suspension, kept on ice, was used for several magnetic measurements over several days. The differential pull under the most favorable conditions, at 10 amperes, is about $\frac{1}{4}$ to $\frac{1}{3}$ of a milligram. This ought to be sufficient to yield fairly reproducible results. In fact, there is a rather large standard deviation of the results. The authors attribute this fact mainly to warm and humid air conditions which are not favorable for precise weighing. A repetition, with a further refinement of the method, is planned for the near future. The present results are shown in Table I.

The result is: the magnetic increment per cc. of the suspension, due to the presence of 0.0596 mg. of Fe, is $(+0.094 \pm 0.017) \times 10^{-7}$. Hence, the susceptibility per g-atom Fe is 8830×10^{-7} . Herefrom the magnetic moment results as follows

$$\begin{aligned}\mu &= 2.84 \sqrt{8830 \times 10^{-7} \times (273 + 27)} \\ &= 4.64 \text{ Bohr magnetons} \pm 0.3\end{aligned}$$

The accuracy of the figure can certainly be improved by further refinements of the method, but even so, the result lends itself to a fair comparison with hemoglobin compounds as obtained by Pauling and Coryell, of which we mention only

Ferro-hemoglobin, Bohr magnetons per Fe atom.....	5.46
Oxy-hemoglobin.....	0 0
Ferri-hemoglobin.....	5.8
Ferri-hemoglobin hydroxide (alkaline methemoglobin).....	4.47

TABLE I

Various cylindrical, double vessels were used, of 6 to 8 mm. outer diameter, and 10 to 13 cm. length of each compartment. The column "Vessel constant" shows the change of volume susceptibility corresponding to a change of one line of differential deflection, according to the calibrations for the particular vessel. All measurements were made at a room temperature of 26 to 27°C. The column "Buffer" gives the deflections in lines, when a phosphate buffer of the same composition, without catalase, was measured. The column " χ " indicates the excess of volume susceptibility of the catalase suspension over that of the corresponding buffer. This is always a positive number, showing that catalase is paramagnetic. No corrections for any change in diamagnetic effect are applied. They are not worthy of consideration at the present state of refinement of the method.

	Buffer	Catalase	Difference	Vessel constant $\times 10^{10}$	" χ " $\times 10^7$
1	-54	-25	+29	4.36	0.126
2	-54	-33	+21	4.36	0.092
3	-84	-41	+43	2.89	0.123
4	-91	-48	+43	2.89	0.123
5	+16.7	+43.5	+26.8	3.15	0.086
6	+15	+39.5	+24.5	3.15	0.077
7	+5	+26.5	+21.5	3.18	0.069
8	+25	+45.8	+20.6	3.18	0.066
9	-18	+18	+36	2.55	0.0915
Average.....					0.094
Probable error.....					± 0.017

The magnetic moment of catalase is distinctly smaller than that of hemoglobin and resembles most that of alkaline methemoglobin.

It is suggestive to investigate also various reaction products of catalase. At the present stage of the investigation we do not wish to give accurate figures yet, but can state that addition of $\text{Na}_2\text{S}_2\text{O}_4$ does not change the susceptibility, which seems to be a corroboration of Keilin's statement that catalase is not reduced by $\text{Na}_2\text{S}_2\text{O}_4$; and that NaCN , and also NaOH , diminish the susceptibility. Since we are working with a suspension, not a solution of the substance, we hesitate to interpret these results for the time being and refrain from giving figures.

CONCLUSION

The magnetic moment of catalase is 4.6 ± 0.3 , nearly the same as that of ferric hemoglobin hydroxide. If the moment be due to electron spin alone without orbital contribution, 3 free electrons would give rise to 3.9 magnetons.

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SOME PROPERTIES OF CRYSTALLINE GUINEA PIG HEMOGLOBIN

By SAM GRANICK

(From the Laboratories of The Rockefeller Institute for Medical Research)

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Several lines of evidence lead to the conception of wet protein crystals as made up of protein molecules with interstices between them that are large and filled with loosely held solvent molecules. Some of the evidence leading to this conclusion may be briefly summarized: (a) the high water content of protein crystals, early noted by Sørensen (1), who estimated that over 20 per cent by weight of egg albumen crystals is water; (b) the ease of water loss under slightly diminished aqueous tension as observed by Katz (2); (c) the change in density when suspended in solutions of different osmotic pressures as studied by Adair and Adair (3); (d) the staining of protein crystals with dyes; (e) x-ray data on wet pepsin crystals, from which Bernal and Crowfoot (4) infer that the protein molecules are relatively dense globular bodies separated by relatively large spaces which contain water.

We wish to present further evidence in support of this idea, and also to describe several other properties of crystalline guinea pig hemoglobin.

I

Action of $K_3Fe(CN)_6$ on Crystalline Oxyhemoglobin.—When a suspension of crystals of guinea pig oxyhemoglobin is treated under the microscope with $K_3Fe(CN)_6$ solution, the red crystals are observed to become yellow-brown while the crystal shape remains intact. (Other oxidizing agents of high potential such as quinone or $KMnO_4$ also bring about this change in color.) With the Abbe microspectroscope the yellow-brown color of the crystals was identified as Mth.¹ This experiment suggested that ferricyanide was penetrating into the crystal to react with the iron in HbO_2 .

In order to determine whether all of the HbO_2 in the crystals could be converted into Mth, a quantitative study with Warburg manometers was made of the O_2 released when HbO_2 is acted upon by ferricyanide. Freshly prepared

¹ Abbreviations used.

Hb = ferrous hemoglobin

HbO_2 = ferrous oxyhemoglobin

Mth = ferri hemoglobin or methemoglobin

washed crystals of guinea pig HbO_2 were suspended in $\text{M}/30$ phosphate buffer pH 7.0 and carefully pipetted into Warburg vessels that had two side arms containing the ferricyanide. The ferricyanide was added in two successive portions. The first portion, 0.05 cc., was sufficient to theoretically react with only

TABLE I

Recovery of O_2 Released by Ferricyanide from Crystals of HbO_2

Each vessel contained 2.0 cc. of HbO_2 crystals* suspended in $\text{M}/30$ Sørensen's PO_4 buffer, pH 7.0 at 29.8°C . (1). The dry weight of HbO_2 per cc. of suspension is 0.0493 gm. \approx 66 c. mm. O_2 when quantitatively converted into methemoglobin with ferricyanide. If recovery of O_2 is 100 per cent then 0.100 cc. of 0.05 M $\text{K}_3\text{Fe}(\text{CN})_6$ should release 112 c. mm. O_2 from HbO_2 .

Vessel No.	0.05 M $\text{K}_3\text{Fe}(\text{CN})_6$ added		O_2 produced		Recovery of O_2
	cc.		c.mm.		per cent
1	0.05		54.6		97.3
	<u>0.10</u>	0.15	<u>72.6</u>	127.2	96.3
1A	0.05		51.8		92.5
	<u>0.05</u>	0.10	<u>53.0</u>	104.8	94.0
3A	0.30		133		100
5A	0.05		54.6		97.5
	<u>0.20</u>	0.25	<u>82.6</u>	137.2	105
7	0.05		53.0		94.6
	<u>0.20</u>	0.25	<u>81.4</u>	134.4	102
8	0.05		57.4		102.5
	<u>0.20</u>	0.25	<u>78.0</u>	135.4	102
1A'	0.20		135.0		102
	+ Veronal sodium†		135.0		102

* The uniformity of pipetting the crystal suspension into Warburg vessels gives rise to a maximum error of 2 per cent, as shown by the dry weight of three samples of HbO_2 suspension pipetted under the same conditions: 0.1190 gm.; 0.1220 gm.; 0.1181 gm.

† 0.20 cc. of a saturated sodium veronal solution was added from the side arm to make the solution sufficiently alkaline to dissolve the crystals. It is seen that no additional oxygen is recovered when the hemoglobin is in solution.

42 per cent of the total oxyhemoglobin. It is seen from Table I that this reaction was quantitative and corresponded stoichiometrically to the amount of ferricyanide added. From Table II it is seen that 85 per cent of the resulting Mth was in the crystalline state at the end of the experiment. Therefore the ferricyanide molecules must have penetrated the crystals and reacted with all the prosthetic groups of oxyhemoglobin. Considering the size and shape

of our crystals, this would mean that some molecules of ferricyanide in order to get to the center of the crystals would have had to penetrate a distance equivalent to the diameter of 1,500 hemoglobin molecules. The conclusion is obvious that there must be interstices in the crystals large enough to permit the movement of ferricyanide into them.

The speed of free diffusion of ferricyanide as calculated from the Stokes-Einstein equation under the conditions of our experiment is approximately 4×10^{-3} cm. per second. It was calculated that if the crystal offered no appreciable resistance to diffusion of ferricyanide, the whole journey through liquid and crystal would take the maximum time of 2 to 3 seconds. The maximum time experimentally determined in the manometer for complete

TABLE II
Solubility of the Crystals Used, at 29.8°C.

To each of two centrifuge tubes with conical ends, add 2.5 cc. HbO_2 crystal suspension in $\text{M}/30 \text{ PO}_4$ buffer pH 7.0. To tube 1 add 0.2 cc. H_2O ; to tube 2 add 0.2 cc. of $0.05 \text{ M K}_3\text{Fe}(\text{CN})_6$. These are kept at 29.8° for 2 hours. They are then centrifuged sharply, the supernatant liquid decanted, and adhering liquid wiped away. Tube 1 now contains crystals of HbO_2 and tube 2 contains crystals of Mth. The crystals were weighed wet, and then dried at 110°C . to constant weight.

Tube No.	Dry weight of Hb in 2.5 cc.	Weight of crystals after decanting		Final amount of Hb suspension in crystal- line state
		Wet	Dry	
1	0.1227 g.	0.2874	0.1097	per cent 89
2	0.1227	0.2790	0.1053	85

release of O_2 was 2 to 3 minutes. Undoubtedly a considerable portion of this time is due to the slowness of equilibration between gas and liquid phase in the manometer vessels. If the ferricyanide ions are to diffuse somewhat readily into the crystals of oxyhemoglobin the interstices must be at least 1.5 to 2.0 times the diameter of the ion, or from 6 to 10 Å.

Let us assume that the hemoglobin molecules are spheres and that they are arranged in closest hexagonal packing. It may be readily calculated that the volume of space between the spherical molecules occupies about 25 per cent of the total volume of the cube, or if filled with water this would mean that 25 per cent by volume or 20 per cent by weight of the wet crystal is water. These are, of course, minimum figures based on the assumption of closest packing and spherical molecules. The distance between the spheres would be just sufficient to permit ferricyanide movement into the spaces. It may be predicted that optically isotropic protein crystals will be found to contain at least this amount of loosely held water between the molecules.

Preparations Used

The tetrahedral crystals of HbO_2 were prepared from guinea pigs, bled by heart puncture into warm sodium citrate solution. The blood was washed twice with warm isotonic saline by centrifugation. To luke the cells three volumes of water were added to one of packed cells. Crystals started to form at once. The laked blood was kept at 37°C . for 2 hours in a 2-inch thick layer in a beaker and then centrifuged slowly for a short time. In this way only the large crystals are deposited, the supernatant solution being discarded. The crystals are suspended in water and centrifuged slowly, the process being repeated two more times. The crystals thus prepared were kept under a thin layer of water at 1°C . overnight. The next morning 10 cc. of the packed crystals were added to 45 cc. of Sørensen buffer ($\text{m}/15$ in phosphate) pH 7.0 and the suspension finally made up to 90 cc. with water. These guinea pig HbO_2 crystals were found to be practically free of catalase activity.

Mth was prepared from HbO_2 crystals by treatment with a slight excess of ferricyanide and subsequently washing the crystals three times by means of centrifuging, and then dialyzing them against water for 24 hours. A solution of Mth was then prepared by making the cold suspension slightly acid with dilute acetate buffer and bringing the solution to neutrality with $\text{m}/15$ disodium phosphate.

II

Location of the Prosthetic Groups.—A corollary of the quantitative oxidation of the hemes by ferricyanide appears to be that the heme groups are on the surface of the globin molecules, otherwise one would have to assume penetration of ferricyanide into the molecules. The latter is made unlikely because of the closeness with which the peptide chains are packed within the molecule. Mirsky (5) has offered this juxtaposition of peptide chains as an explanation for the non-detection of $-\text{SH}$ groups in some native proteins by means of ferricyanide. The data presented in Table I show that the hemes react quantitatively with the ferricyanide added. If some ferricyanide had oxidized $-\text{SH}$ groups before it oxidized the heme groups then a lower O_2 production per ferricyanide molecule would have been expected. For example, assuming only one free $-\text{SH}$ group reacting per hemoglobin molecule, one would expect to recover only 75 per cent of the O_2 .

In addition to being localized on the surface of the globin, the four hemes per globin must be facing the spaces between the molecules, or in other words, the hemes are not the points of closest contact between the molecules.

III

Further Evidence of Permeability of HbO_2 Crystals.—Hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$) may also be shown to penetrate the crystal of HbO_2 removing the oxygen and producing Hb. This becomes evident by diminishing the solubility of Hb with ammonium or sodium sulfate. When HbO_2 crystals were suspended in 50 per cent saturated ammonium sulfate and hydrosulfite added, the crystals could be seen to turn violet under the microscope and the Hb band became

visible at the same time that the HbO_2 bands vanished in the micro spectro-scope. These violet crystals go into solution within a few minutes. If the concentration of ammonium sulfate is increased to near saturation many of the purple crystals do not dissolve.

The staining of protein crystals with dyes may also represent penetration of the dye molecules into the crystal spaces. Several dyes in aqueous solution were added to HbO_2 crystals and examined microscopically. Due to the intense color of the crystals it is difficult to judge their penetration. Crystal violet, however, stained the crystals intensely but not methyl green. Catalase prisms and plates were better objects because of their negligible color (6). Methylene blue and toluidine blue stained them faintly, crystal violet very strongly, and methyl green very faintly. If the dye concentration, is properly chosen the crystals will become deeply colored and the solution colorless. The staining is not merely a surface adsorption since thicker crystals take up a very much deeper stain than thinner ones.

IV

The Relative Solubilities of Hb, HbO_2 , and Mth.— HbO_2 , as isolated from guinea pig blood is rather insoluble at room temperature. A suspension kept in $\text{M}/30 \text{ PO}_4$ buffer, pH 7.0, for 1 day at 1°C . and then for 3 hours at 30° was 89 per cent in the crystalline state. Mth is slightly more soluble, 85 per cent remaining in the crystalline state under these conditions. Hb, however, is very soluble at this pH.

When crystals of HbO_2 are watched under the microscope, as hydrosulfite diffuses to them, they are seen to melt away rapidly while still retaining their tetrahedral shape. At the same time the Hb band becomes visible. A thick suspension of HbO_2 crystals is a muddy red-brown. When treated with hydrosulfite it becomes a clear deep purple; on shaking with air it gradually becomes red, then cloudy, and begins to deposit crystals all within 1 to 2 minutes. Here is an example, then, of an aggregation and dissolution brought about by the mere addition or removal of oxygen.

V

The O_2 - HbO_2 Equilibrium in Crystalline HbO_2 .—The removal of O_2 from a solution of HbO_2 may be readily accomplished by lowering the pressure of O_2 . Under our conditions of evacuation from a Thunberg tube with an oil pump, it required less than 2 minutes to completely convert the dilute HbO_2 solution to Hb. However, if a suspension of HbO_2 crystals is treated in the same manner as the HbO_2 solution, then no appreciable O_2 can be removed even after 15 minutes of constant pumping, although the pressure was 15 mm. to 5 mm. of mercury (decreasing as the temperature of the suspension decreased due to evaporation).

The following experiments show that the oxygen of HbO_2 crystals can be

removed only by considerably decreasing the oxygen tension: (a) Bubbling either H_2 or N_2 gases (free from O_2) through a suspension of HbO_2 crystals at 30° for 20 to 30 minutes completely converted them to Hb solution. (b) Removal of O_2 by hydrosulfite: HbO_2 crystals in $m/15$ PO_4 buffer pH 7 were placed in a Thunberg tube and evacuated. A minute amount of hydrosulfite was then added from a side arm. Within 1 to 2 minutes the suspension became a solution of Hb. The pH after the experiment was 6.9. (c) Removal of O_2 by activated hydrogen: A suspension of HbO_2 crystals in the presence of traces of methylene blue or catalase was treated with H_2 + colloidal Pd. Within 1 to 2 minutes this suspension had been completely converted to a solution of Hb. (d) Replacement of O_2 by CO: A suspension of HbO_2 crystals was treated with pure CO gas for 5 minutes. The CO replaced the O_2 in the crystals without changing their shape, since on the addition of a small amount of hydrosulfite no Hb results and the crystals remained intact. On the addition of a large excess of hydrosulfite, the crystals began to dissolve very slowly and unevenly, this being due to a decrease in pH.

The firmer binding of O_2 in HbO_2 crystals, as contrasted to HbO_2 in solution, may indicate that the coordination of the molecule in the crystal has diminished the competition of other groups than O_2 for the iron. The HbO_2 crystals can be readily dissolved, away from the isoelectric point, and in the dissolved state the HbO_2 molecules readily give up their oxygen on diminishing the O_2 tension. The firm binding of O_2 in HbO_2 crystals would obviously prevent hemoglobin in the crystalline state from functioning as a readily reversible oxygen carrier.

VI

Action of H_2O_2 on HbO_2 .—The crystals of HbO_2 of the guinea pig, crystallized once, are practically free from catalase. On addition of a few drops of 0.5 per cent H_2O_2 to a suspension of HbO_2 crystals, the two prominent bands at 578 and 540 become faint, the 578 band appears to broaden, spread out to 590, and is relatively stronger than the 540 band, while the original crystalline shape remains unchanged. This compound has the same absorption bands as that of H_2O_2 -methemoglobin solution first described by Keilin and Hartree (7).

VII

Action of Activated Hydrogen on Mth.—Methemoglobin either in solution or in crystalline suspension at pH 7.0 in PO_4 buffer is very slowly reduced by activated hydrogen. Mth plus a trace of catalase and caprylic alcohol was bubbled with hydrogen for 3 minutes and then 2 drops of colloidal Pd added. The bubbling was continued for 15 minutes during which time only traces may have been reduced as observed spectroscopically in comparison with the control.

This slow interaction may be partially explained by the fact that we are dealing with two colloids. If a trace of methylene blue or rosindulin GG is

added to the Pd-H₂ solution containing Mth plus a trace of catalase, then reduction to ferrohemoglobin is complete in 1 minute in the case of the solution, and reduction and solution of Mth crystals is complete in 2 to 3 minutes. The methylene blue molecules being small and reversibly oxidized act as intermediary between the two colloids.

This is in agreement with the fact that also the electron exchange between hemoglobin and the bright Pt electrode is so sluggish that potentials are only poorly established.

VIII

The Action of Activated Hydrogen on HbO₂.—One of the intermediates produced in the reduction of O₂ either as such or in the form of HbO₂, by activated Pd-H₂ is H₂O₂. This may be shown by the following experiment. HbO₂ either in solution or as crystal suspension in PO₄ buffer pH 7, was treated with Pd-H₂. Within 45 seconds a band at 630 mμ had arisen and also the H₂O₂-Mth bands had appeared. The 630 band was identified as Mth by forming the Mth-NaF compound. If a trace of catalase is added to the HbO₂ solution, then Pd-H₂ added, the Hb is formed within 1 minute and in the case of the crystalline HbO₂, removal of O₂ and solution of crystals occurred in 1 to 3 minutes. No Mth band was observed.

Instead of using catalase to destroy the H₂O₂, a trace of methylene blue was used with identical results. Within 3 seconds after PdH₂ was added, the dye was decolorized and within 20 to 40 seconds the HbO₂ bands had vanished. Methylene blue may act here in three ways. Its reduced form may be oxidized by H₂O₂ thus destroying some peroxide; it may act on any Mth that might have arisen from the action of peroxide on Hb; and it may react with the minute amount of O₂ in solution.

SUMMARY

1. Guinea pig hemoglobin crystals are shown to be readily permeable to ferricyanide and hydrosulfite, indicating the presence of interstices between the protein molecules of the crystal.

2. The assumptions of closest hexagonal packing and of spherical molecules of HbO₂ lead to a crystal lattice having interstices between the molecules which represent 25 per cent by volume of the crystal. These spaces would be just large enough at their narrowest junctures to permit ferricyanide ions to diffuse through them. If these spaces were filled with water, then 20 per cent by weight of the crystals would be water.

3. The hemes are on the surface of the globin and are arranged facing the interstices between the molecules of the lattice.

4. The binding of O₂ in HbO₂ is stronger in the crystal lattice than in solution.

5. Hydrogen, activated with colloidal palladium, will not reduce ferrihemoglobin except in the presence of a redox dye.

6. In the reduction of O_2 by activated hydrogen, H_2O_2 can be demonstrated by the formation of the H_2O_2 -ferrihemoglobin spectrum.

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IMMUNE RESPONSE OF MICE TO ACTIVE VIRUS AND TO FORMALIN-INACTIVATED VIRUS OF EASTERN EQUINE ENCEPHALOMYELITIS

By ISABEL M. MORGAN AND PETER K. OLITSKY

(From the Laboratories of The Rockefeller Institute for Medical Research)

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This study on the immune response of mice to the virus of Eastern equine encephalomyelitis is divided into two parts. The first deals with a comparison of the duration of active immunity, induced by (a) active virus and (b) formalin-inactivated virus. The second is concerned with the correlation of cerebral resistance with serum-neutralizing antibody.

I. DURATION OF ACTIVE IMMUNITY

It is generally recognized that many viruses can be inactivated by formalin, and that a number of viruses when so inactivated may retain their immunizing capacity.

Successful immunization has been achieved in natural and in experimental infections by use of formalin-inactivated virus: among natural diseases in cattle, foot and mouth disease (1) and cattle plague (2); in horses, Eastern and Western equine encephalomyelitis (3) and in dogs and ferrets, distemper (4). By use of formalin-inactivated virus, protection against experimental diseases has been accomplished, as with herpes in guinea pigs and with psittacosis in mice (5). A complex vaccine of formolized distemper and influenza viruses induced in ferrets active immunity and neutralizing antibodies against influenza virus (6). By means of choriomeningitis virus inactivated by formalin, guinea pigs were rendered resistant to injection of active virus and developed neutralizing antibodies in their serum (7). In each case it has been found that considerably more formalin-inactivated virus than active virus must be injected to bring about an equal degree of immune response.

In some instances, on the other hand, virus so inactivated brought about questionable or a low degree of resistance, although antibodies were demonstrable. Thus formolized elementary bodies of vaccinia were found to be weakly antigenic in rabbits, but by means of repeated injections, neutralizing antibodies were produced as well as a low degree of resistance to infection with active vaccine virus, which was not as enduring, however, as that brought about by active virus (8). Several investigators agree that formolized poliomyelitis virus could bring about neutralizing antibodies but differ on the question whether it could give rise to resistance to active virus injected intracerebrally (9). Attempts to demonstrate an immune response in rhesus monkeys with formalin-inactivated virus of yellow fever were unsuccessful (10).

To what extent formalin-inactivated virus, in sufficient dosage, can reproduce the antigenicity of active virus may be determined by the following criteria: (1) titer of serum-neutralizing antibody induced, (2) degree of active immunity, and (3) duration of active immunity.

With respect to the first criterion, it has already been demonstrated (11) that by sufficient immunization with formalin-inactivated E.E.E. virus an antiserum could be produced which, by all tests applied, equalled, in antibody-concentration, antiserum induced by active virus. This will be discussed at greater length in the next section of this paper. As for the second point concerning the degree of active immunity, it has been previously reported (12) that by sufficient vaccination with formalin-inactivated virus, as with active virus, cerebral resistance can be induced to as much virus as is possible to inject; i.e., of the order of 10,000,000 cerebral units. The first section is concerned with the third point of whether the immunity induced by formalin-inactivated virus is as enduring as that by active virus. Cox and Olitsky (3) found that 3 months after vaccination of guinea pigs with active, or with inactivated virus, cerebral resistance was equally solid; from 3 to about 7 months, the resistance diminished in both cases.

MATERIALS AND METHODS

2-months-old albino mice, Rockefeller Institute strain, were vaccinated as follows: one large group was vaccinated by means of 2 intra-abdominal injections of 0.25 ml 10^{-8} dilution in broth of E.E.E. virus-infected mouse brain (A.V.) at a week's interval. This method of vaccination was chosen since it was known that the majority of mice would survive and would develop a high degree of immunity. Of 109 mice injected, 15 died during the week following the first injection. Mice in another large group received 6 intra-abdominal injections of 0.25 ml 10^{-1} of a suspension of virus-infected mouse-brain which had been inactivated with 0.5 per cent formalin (38 per cent formaldehyde); this preparation is designated F.V. Injections were given on the 1st, 2nd, 3rd and 8th, 9th, 10th days. Details of the procedure have already been described (13). Repeated injections of formalized virus were known to be required for development of a high degree of resistance (11). One-fifth of each group of mice was tested for resistance by intracerebral injection of 0.03 ml of broth dilutions of virus-infected mouse-brain at the following intervals after beginning of vaccination: 2 weeks, 2, $4\frac{1}{2}$, and $6\frac{1}{2}$ months. At 6 months, an additional single dose of active or formalin-inactivated virus was injected intra-abdominally into half of each of the remaining groups. At the $6\frac{1}{2}$ months' test for resistance, there were thus two additional groups which had received a single "step-up" dose of antigen 2 weeks previously. In order to control the effect of this "step-up" dose, two groups of normal mice, more than 6 months of age, were injected with a similar, single dose of active or formalin-inactivated virus; they were tested for cerebral resistance 2 weeks after vaccination. All animals were bled from the tail the day before test for cerebral resistance. A study of neutralizing antibody will be reported in the next section.

Experimental

The degree of cerebral resistance found at the intervals indicated is recorded in figure 1.

At 2 weeks after beginning of vaccination, A.V.-vaccinated mice (group A, figure 1) showed cerebral resistance of 10,000,000 units, as measured by difference between cerebral titer of virus in the non-vaccinated control group and that in the vaccinated group. F.V.-vaccinated mice at 2 weeks (group B) presented a solid immunity, a resistance of at least 100,000,000 cerebral units. Thus once more it has been shown that formalin-inactivated virus in sufficient dosage can

Duration of Cerebral Resistance of Mice at Intervals
after Vaccination with Active or Formalin-Inactivated E.E.E. Virus

Vaccination	2 weeks	2 months	4 $\frac{3}{4}$ months	6 $\frac{1}{2}$ months	6 $\frac{1}{2}$ mos.+2 wks	2 weeks
AV. ↑ ↑	A	C	E	G	↑ I	↑ K
FV. ↑↑↑ ↑↑↑	B	D	F	H	↑ J	↑ L
Virus dilution						
10 ⁻¹	■ ■ ■ ■					
10 ⁻²	■ ■ ■ ■	■ ■ ■ ■				
10 ⁻³	■ ■ ■ ■	■ ■ ■ ■				
10 ⁻⁴	■ ■ ■ ■	■ ■ ■ ■	■ ■ ■ ■		■ ■ ■ ■	■ ■ ■ ■
10 ⁻⁵		■ ■ ■ ■	■ ■ ■ ■	■ ■ ■ ■	■ ■ ■ ■	■ ■ ■ ■
10 ⁻⁶			■ ■ ■ ■	■ ■ ■ ■	■ ■ ■ ■	■ ■ ■ ■
10 ⁻⁷			■ ■ ■ ■	■ ■ ■ ■	■ ■ ■ ■	■ ■ ■ ■
10 ⁻⁸			■ ■ ■ ■	■ ■ ■ ■	■ ■ ■ ■	■ ■ ■ ■
				■ ■ ■ ■		■ ■ ■ ■
Non-vaccinated control mice						
10 ⁻⁶			■ ■ ■ ■		■ ■ ■ ■	
10 ⁻⁷	■ ■ ■ ■	■ ■ ■ ■	■ ■ ■ ■		■ ■ ■ ■	■ ■ ■ ■
10 ⁻⁸	■ ■ ■ ■	■ ■ ■ ■	■ ■ ■ ■		■ ■ ■ ■	■ ■ ■ ■
10 ⁻⁹	■ ■ ■ ■	■ ■ ■ ■	■ ■ ■ ■		■ ■ ■ ■	■ ■ ■ ■

■=1 mouse died □=1 mouse survived

Fig. 1

reproduce the antigenicity of active virus by inducing at least as high a degree of cerebral resistance.

At 2 months after beginning of vaccination, only half of each group of mice, whether F.V.- or A.V.-vaccinated, resisted the injection of virus over a wide range of dilutions. This resistance of half the number of individuals, almost regardless of the amount of virus injected, has been recognized before (13) as characteristic of an intermediate degree of group-resistance, in contrast to the clear-cut endpoints obtained when resistance is either high or low. At 2 months,

then, the F.V.-vaccinated group had retained immunity equally as well as the A.V.-vaccinated. At 4½ months resistance had fallen to a low level in A.V.- as well as in F.V.-vaccinated mice (groups E and F). However, a small, though measurable amount of resistance was still present in each group 6½ months after vaccination (groups G and H). After these intervals, the F.V.-vaccinated mice showed at least as much resistance as the A.V.-vaccinated. When tested at 6½ months, mice of groups I and J, which had received 2 weeks previously a "step-up" dose of active or formalin-inactivated virus, presented a solid resistance to the amounts of virus injected, but they were not tested with maximal amounts. Groups K and L served to control the effect of a single dose of either antigen. Mice in groups K and L were over 6 months of age, and were therefore approximately the same age as those in groups I and J. A single intra-abdominal injection of active virus induced at least a moderate degree of immunity when tested after 2 weeks (group K), but no endpoint was reached. However, this group was not solidly immune as was group I, which had had the benefit of vaccination 6 months previously. Group L, 2 weeks after a single injection of F.V., showed little resistance in contrast to group J, solidly resistant as far as tested, which had the advantage of the earlier vaccination. The difference between group K and group L brings out clearly that active virus is a far better antigen than formalin-inactivated virus when compared on the basis of a single injection of each. The immune response to active virus was of a higher degree than that to the formalized vaccine, in spite of the fact that 100 times as much virus was contained in the dose of the latter.

Thus, although at the end of a 6 months' period, A.V.- and F.V.-vaccinated mice retained only a low degree of cerebral resistance, this resistance could be raised by a single injection of either antigen to a level above that induced by such a single injection in normal mice.

To conclude, it has been shown that mice could be immunized by means of a sufficient dosage of formalin-inactivated virus to as high a level of cerebral resistance as that induced by active virus. Furthermore, cerebral resistance was found to endure after vaccination with either antigen for at least 6 months, although at a low level. At that time, mice previously vaccinated were capable of giving a greater immune response to a single injection of either antigen than were non-vaccinated mice.

II. CORRELATION OF SERUM-NEUTRALIZING ANTIBODY WITH CEREBRAL RESISTANCE

The sera taken at intervals after vaccination with active virus or formalin-inactivated virus were studied and compared for neutralizing capacity. Since these sera were obtained over a period during which cerebral resistance of mice rose rapidly in response to vaccination, then gradually declined to a low level,

and once more rose in response to further antigenic stimulus, they were well suited to a study of correlation of neutralizing antibody with rise and fall of cerebral resistance.

Experimental

Titration of neutralizing capacity of the sera of A.V.- and F.V.-vaccinated mice was carried out by the serum-dilution method; that is, dilutions of test-sera in saline solution, as shown in figure 2, were mixed with equal volumes of 2×10^{-7} dilution of active virus. 0.03 ml of each mixture was injected intracerebrally into 4 young adult mice. To control the amount of virus thus injected, virus-suspension in dilutions of 2×10^{-7} , 2×10^{-8} and 2×10^{-9} was titrated intracerebrally in normal mice in groups of 4 or 8; in each test, normal

Titration of Neutralizing Antibody:
Neutralization of 10 Units of E.E.E. Virus by Dilutions of Serum of Mice
at Intervals after Vaccination with Active or Formalin-Inactivated E.E.E. Virus

Serum of mice		2 weeks	2 months	4¾ months	6½ months	6½ mos + 2 wks	2 weeks	
AV-vaccinated		A	C	E	G	I	K	
FV-vaccinated		B	D	F	H	J	L	
Dilution of serum	Final dilution of virus							
1:10,000	10 ⁻⁷	■■■■				■■■ ■■■		
1: 3,000		■■■ ■■■				■■■ ■■■		
1: 1,000		■■■ ■■■	■■■ ■■■	■■■ ■■■		■■■ ■■■	■■■ ■■■	
1: 300		■■■ ■■■	■■■ ■■■	■■■ ■■■	■■■ ■■■	■■■ ■■■	■■■ ■■■	
1: 100		■■■ ■■■	■■■ ■■■	■■■ ■■■	■■■ ■■■	■■■ ■■■	■■■ ■■■	
1: 30		■■■ ■■■	■■■ ■■■	■■■ ■■■	■■■ ■■■	■■■ ■■■	■■■ ■■■	
1: 10						■■■ ■■■	■■■ ■■■	■■■ ■■■
Virus titrations								
Normal serum	10 ⁻⁷	■■■■■■■■	■■■■■■■■	■■■ ■■■ ■■■	■■■■■■■■	■■■■■■■■	■■■■■■■■	
	10 ⁻⁸	■■■■■■■■	■■■■■■■■	■■■ ■■■ ■■	■■■■■■■■	■■■■■■■■	■■■■■■■■	
	10 ⁻⁹	■■■■■■■■	■■■■■■■■	■■■ ■■■ ■■	■■■■■■■■	■■■■■■■■	■■■■■■■■	

■=1 mouse died □=1 mouse survived

FIG. 2

serum was diluted to the lowest dilution of test-serum. Whenever possible test-sera were compared simultaneously. Final dilutions of virus-suspension are given in figure 2, whereas serum-dilutions must be multiplied by one-half to give final dilutions.

From titrations of virus with normal serum shown in figure 2, it may be seen that the titer of virus was 10^8 or higher in each test; that is, from one-half to all of each group of mice injected with 10^{-8} dilution of virus died. In order that 10^{-7} dilution of virus injected with test-sera should represent 10 units of virus, a corresponding endpoint was selected, of death of more than half the number of mice in a group. Thus, titer of an antiserum was taken as the dilution of serum at which 3 of 4 test-mice died.

Figure 2 shows that at 2 weeks the antibody-titer of serum B, from F.V.-vaccinated mice, was fully as high as, or slightly higher than that of serum A, from A.V.-vaccinated mice.

It should be recalled that a far greater dosage of formalin-inactivated virus was used in vaccination. Nevertheless, it has been shown

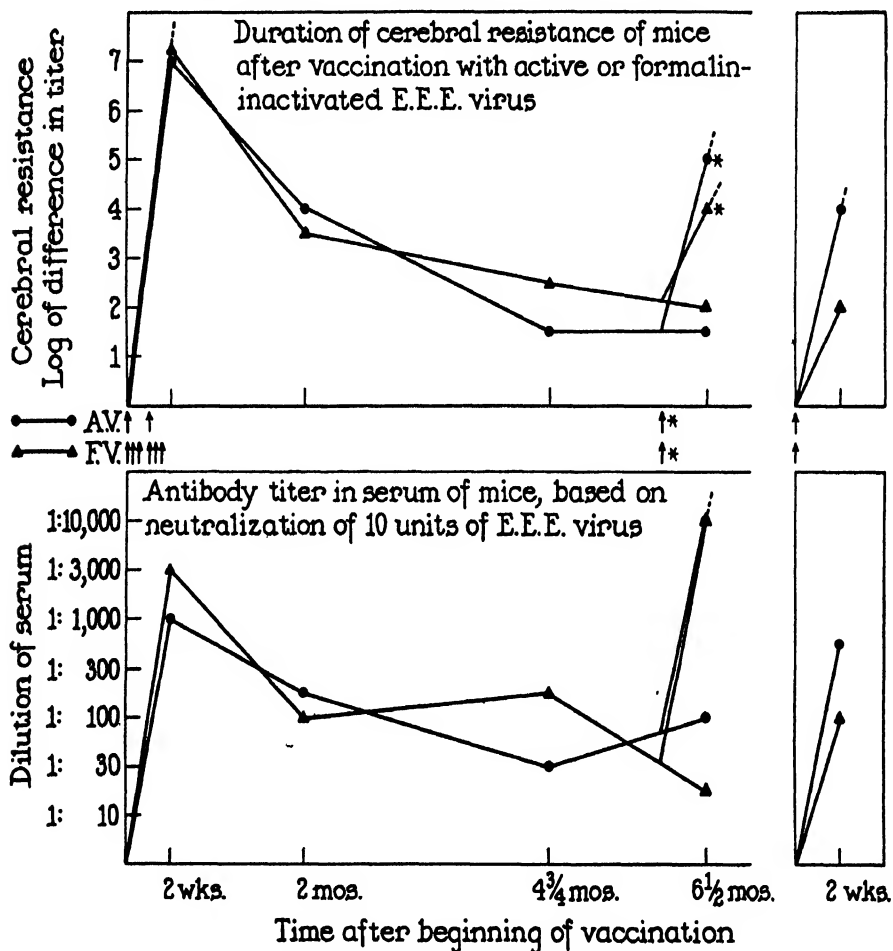


FIG. 3

again (11) that sufficient vaccination with formalin-inactivated virus could bring about as great an antibody response as that induced by active virus. Antibody-concentration fell in both types of antisera from 2 weeks at 6½ months (sera A and B through G and H). The "step-up" dose of either antigen raised the antibody-titer (sera I and J) to the highest level attained, greater than 1:10,000

dilution of serum, and considerably higher than that of serum of mice (groups K and L) which received only a single dose of each antigen.

Correlation of antibody and cerebral resistance. When titer of antibody in serum of vaccinated mice was followed over a period of more than 6 months, it was found to rise rapidly, and then fall, gradually to a low level; however, as long as 6 months after vaccination, far greater antibody-response to a single antigenic stimulus was given by previously vaccinated mice than by non-vaccinated mice. This rise and fall and subsequent rise (on further stimulation) of neutralizing antibody reflected the cerebral resistance of the vaccinated mice, described in the previous section. In figure 3, a comparison of cerebral resistance with serum antibody-titer in vaccinated mice is presented.

Since, as shown in figure 3, each rise or fall in cerebral resistance was paralleled by rise or fall in serum-neutralizing antibody, a correlation has been demonstrated between degree of cerebral resistance and titer of serum-neutralizing antibody.

DISCUSSION

At one time there was some perplexity as to an apparent difference between the antibody to formalin-inactivated virus of Eastern equine encephalomyelitis (F.V.) and that to active virus (A.V.) when antisera prepared in several species of animals were compared by serum-neutralization tests. Undiluted sera were compared on the basis of amount of virus neutralized. When serum-virus mixtures were injected into mice by the intracerebral route, F.V.- and A.V.-antisera protected against equal, or almost equal, amounts of virus. When, however, the neutralization-test was carried out by the intra-abdominal route, A.V.-antisera protected against far greater quantities of virus than did F.V.-antisera. This apparent discrepancy cleared up when the two types of antisera were compared on the basis of dilution of serum, that is, by a true titration of antibody (11). The result was that the A.V.-antisera could be diluted as much as one hundredfold and still protect against as much virus as undiluted serum could. In contrast, F.V.-antiserum on dilution could no longer protect by the intracerebral route to the same degree. In other words, although antisera appeared equal by cerebral test, there was actually an excess of antibody in one serum (A.V.) which, however, was not demonstrable by the use of undiluted sera. Thus the apparent inequality of the sera was in reality an indication of the limit of the cerebral neutralization-test. By the intra-abdominal test, the real difference in concentration of antibody in sera was shown. The difference was therefore quantitative and indeed, by further immunization, F.V.-antiserum could be produced which equalled A.V.-antiserum, as shown by the intra-abdominal neutralization-test, as well as by titration of antibody by means of

dilution. This experience indicated that serum-dilution rather than virus-dilution is the method of choice for comparison of sera on the basis of antibody-concentration.

Howitt found no correlation in guinea pigs between humoral antibodies and tissue-immunity in studies on equine encephalomyelitis virus Western strain (14). With the Eastern strain, Cox and Olitsky (15) demonstrated that a high degree of tissue-immunity was associated with a minimal degree of serum-antibody; moreover, guinea pigs vaccinated insufficiently to develop cerebral resistance showed no neutralizing antibodies. Olitsky and Harford (16) interpreted later results as consistent with the hypothesis that the content of antibody is not proportional to the degree of resistance to infection. In all these studies the neutralization-tests were carried out with undiluted serum. In the present study little differentiation between neutralizing capacity of the sera was found by the usual cerebral neutralization-test when undiluted serum was mixed with tenfold dilutions of virus. Only when a true titration of antibody was carried out, by serum-dilution in lower multiples, could the neutralizing capacity of the sera be widely differentiated.

The limitation of tests with undiluted serum was apparent in an earlier study of mice of various age-groups vaccinated with formalin-inactivated virus of Eastern equine encephalomyelitis (12). Although beyond a certain age, immune response in terms of neutralizing capacity of their undiluted sera could not be widely differentiated, yet there was a marked difference in degree of cerebral resistance. With other viruses causing encephalitis, for example, rabies virus, a similar influence of age on immune response of mice has been reported (17). Sera of vaccinated mice of various ages could not be differentiated on the basis of amount of virus neutralized, although cerebral resistance increased with age. For rabies as well as equine encephalomyelitis virus (13); the presence of small amounts of antibody as a result of vaccination does not insure cerebral resistance; on the other hand, when cerebral resistance is present in vaccinated animals, serum-antibodies can always be demonstrated and, furthermore, have been shown here to correlate, in the case of the equine virus. A previous study has already demonstrated a correlation of antibody and resistance to peripheral injection of Eastern equine encephalomyelitis virus in vaccinated mice (13). In immunization with St. Louis encephalitis virus, however, where an inverse correlation between cerebral resistance and neutralizing antibody over a period of time has been reported (18), it is not possible to apply such an explanation.

Repeated vaccination of man against the virus of equine encephalomyelitis has been carried out by Beard, Finkelstein and Beard (19) by injection of formalin-inactivated chick-embryo virus. Antibodies present to a high degree 2 weeks after vaccination were found to have diminished to an intermediate or low level, depending on the strain of virus, by 6 to 9 months after vaccination.

Response to revaccination with a single dose was markedly greater than that after the initial vaccination, a reaction similar to that in mice as reported here. Experimental evidence is now offered for their assumption that vaccination induces a certain degree of active immunity, reflected by the development of serum-neutralizing antibody. The relation of antibody to such induced immunity has been discussed.

SUMMARY

It has been shown that mice could be immunized by means of a sufficient dosage of formalin-inactivated virus of Eastern equine encephalomyelitis to at least as high a degree of cerebral resistance as that induced by active virus. Cerebral resistance, high at 2 weeks after vaccination of both groups, fell gradually over a period of 6 months; it was once more raised to a high degree by further antigenic stimulus.

Mice vaccinated with a sufficient dosage of formalin-inactivated virus had developed at 2 weeks fully as high a concentration of serum-neutralizing antibodies as mice vaccinated with active virus. The concentration fell in both groups during the following 6 months. At the end of that time, mice injected with a single dose of either active or inactivated virus gave a greater antibody-response than after the initial course of vaccination.

Since each rise and fall of cerebral resistance of vaccinated mice was reflected by similar rise and fall of serum-neutralizing antibody, a correlation has been demonstrated between titer of antibody and degree of cerebral resistance.

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THE ENDURING PARTNERSHIP OF A NEOPLASTIC VIRUS AND CARCINOMA CELLS*

CONTINUED INCREASE OF VIRUS IN THE V2 CARCINOMA DURING PROPAGATION IN VIRUS-IMMUNE HOSTS

By JOHN G. KIDD, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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The rabbit papilloma virus (Shope) gives rise under natural conditions to horny cutaneous growths in wild cottontails (1), and to papillomas of similar sort when inoculated into domestic rabbits. The growths of both species may become malignant after a while through alterations in the virus-infected cells (2); but the virus has never been recovered from the cancers, nor can it be got ordinarily from many of the papillomas induced with it in domestic rabbits. Yet it persists in masked or altered form in the cancers as well as in the papillomas, as serological tests have proved: an antibody directed specifically against the virus appears in the blood of rabbits carrying the growths, and its titer rises as the tumors enlarge (3).

One of the cancers originating in a virus-induced papilloma and transplanted successfully—the V2 carcinoma—has now been carried through 21 tumor generations in the course of more than three years. It grows rapidly in a considerable proportion of hosts and often metastasizes. Tests have been made for the specific antiviral antibody in the blood of rabbits of every tumor generation,—more than a hundred animals in all. This has never been found in the blood of normal control animals, nor in noteworthy titer in rabbits implanted with the tumor but negative; but it has regularly appeared in the blood of rabbits in which the V2 carcinoma has grown progressively, attaining a titer as high as that in animals which have long carried large papillomas, or even higher. The strength of the antibody has been as great in recent tumor generations as in the early ones (3).

The persistence of virus in the transplanted V2 carcinoma does not seem remarkable when the fact is recalled that wholly extraneous, parasitic viruses—vaccinia, virus III, yellow fever, infectious ectromelia, to name but a few—can ride along as passengers in other transplanted tumors (4), as can also spirochetes, pleuropneumonia-like microorganisms, and various other bacteria (5).

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But the fact is now becoming manifest that passenger viruses may disappear spontaneously from the tumors they ride upon, while some have been got rid of by transplanting the growth to hosts immune to the virus. Rivers and Pearce observed long ago that virus III could vanish from the Brown-Pearce tumor as casually as it had appeared, and Andrewes recently noted that a rabbit sarcoma—the RSI—became free of passenger virus III when transplanted through a rabbit previously immunized against it (6). He also found that the Brown-Pearce carcinoma could be rid of virus III by propagation in two successive virus III-immune rabbits, and from vaccine virus by passage through a single animal immune to it. Identical results were got with the same materials by Pearce (7).

These findings bring up the possibility that the V2 carcinoma can be freed of associated virus by growing it in a succession of hosts immunized against this. The work here reported was done to learn whether such is the case. The general plan was to propagate the V2 carcinoma in five successive groups of animals all hyperimmunized beforehand against the papilloma virus, and then to determine whether, upon return to normal hosts, the growth would elicit antiviral antibodies in the same high titer as before.

Methods

To *hyperimmunize* the rabbits to which the tumor was to be transferred, four “pancake” papillomas were produced on the flanks of the animals by rubbing a potent virus suspension into areas of skin about 6×8 cm. which had been freshly scarified with sandpaper. Confluent papillomatosis usually resulted after an incubation period of about 10 days, and the growths rapidly enlarged into characteristic horny masses, these often rising 2 cm. or more above the skin level. Growths of this sort usually elicit antiviral antibody in considerable titer, and this can be greatly increased by repeated injections of large quantities of virus intraperitoneally, as previous work has shown (8). Accordingly, 10 days to 3 weeks after the scarifications, 10 cc. of a 1:20 Berkefeld V filtrate of wild rabbit papillomas which were known to contain virus in quantity was injected intraperitoneally into each animal. The intraperitoneal injections were repeated 7 days later, and after a further interval of 7 to 10 days the rabbits were bled from an ear vein and the sera tested. At this time they were implanted with the tumor.

Much use was made of the *complement fixation test* to titer the antiviral antibody. Previous work had demonstrated its specificity and reliability as a gauge of immunity to the papilloma virus (9). The test was carried out as previously described (9), using 2 units of complement (titrated immediately beforehand) and 2 hours at room temperature for fixation. Antigen from two sources was used—the natural papillomas of W. R. (wild cottontail rabbit) 1-28, and the pooled natural papillomas of five cottontails, P. Both materials yielded much virus. A 1:120 saline extract of the glycerolated papillomas from both sources had been found in previous experiments to provide an optimal quantity of antigen (virus), and hence this dilution was uniformly used. Mention need hardly be made here of the fact that the complement

fixation reaction provides a comparative, not an absolute, measure of the titer of antiviral antibody. Sometimes the results with a single serum varied by as much as a twofold dilution in different tests; but the variation was never greater. A serum can be considered very potent if, after dilution to 1:24 or more, it contains enough antibody to fix completely 2 units of complement in mixture with an optimal dose of antigen. Such a serum is capable of neutralizing many thousand infectious doses of virus, and an animal yielding it is usually resistant to infection with the virus, as previous studies have shown (8). In the present work the antibody titer has been expressed in terms of the highest dilution of serum that gave complete or almost complete fixation in the standard test. Dilutions beyond 1:128 were not tested, for when fixation is complete at this dilution the quantity of antibody is great, and hyperimmunization may be deemed maximal.

The *neutralization test* for antiviral antibody was also used. It was applied as previously described (14, 9).

The *virus suspensions* for primary inoculation and subsequent hyperimmunization were made by grinding the glycerolated natural papillomas of cottontails in a mortar with sand and suspending the ground paste in physiological saline, usually 1:10 to 1:20 (10 per cent and 5 per cent extracts). The suspension was then spun clear, and sometimes passed through a Berkefeld V filter in addition. To spread the conditions, five different virus materials were used, all highly pathogenic. Always a different material was injected intraperitoneally from that with which the rabbit had been cutaneously inoculated.

Normal adult gray brown rabbits (agouti hybrids) were used throughout, males and females indiscriminately. All weighed 2.5 kilos or more.

Transplantation of the tumor was effected by a method somewhat different from that used in the previous work, in order to provide conditions under which the antiviral antibody might have better opportunity to act. A fine suspension of carcinoma tissue was used instead of hashed pieces, and the tissue was kept moist and finally suspended in Tyrode to which immune serum had been added. The tumors were procured with aseptic precautions, and the "healthiest" portions carefully selected from nodules taken from at least two different situations in each animal. By means of a small pestle, the tumor tissue was pressed through a 40-mesh monel metal sieve, with the addition of Tyrode-immune serum (usually 10 parts of Tyrode to one of the animal's own serum), the result being a turbid, finely particulate suspension. 1 cc. portions of the suspension were then implanted into the leg muscles of the new hosts at six situations,—both forelegs and the anterior and posterior muscles of both thighs. The fastest growing tumors were sometimes selected for transplantation and again slower-growing ones were utilized to spread the conditions.

Successive Transplantations of the V2 Carcinoma in Virus-Immune Rabbits

The course of the transplantations is summarized in Chart 1.

The Starting Tumor.—The history of the V2 carcinoma has already been given in detail from its inception to the 13th serial transplantation, when the present experiments were begun (3). To procure material for them a 12th generation tumor was implanted into the muscles of the upper forelegs and anterior and posterior thighs—

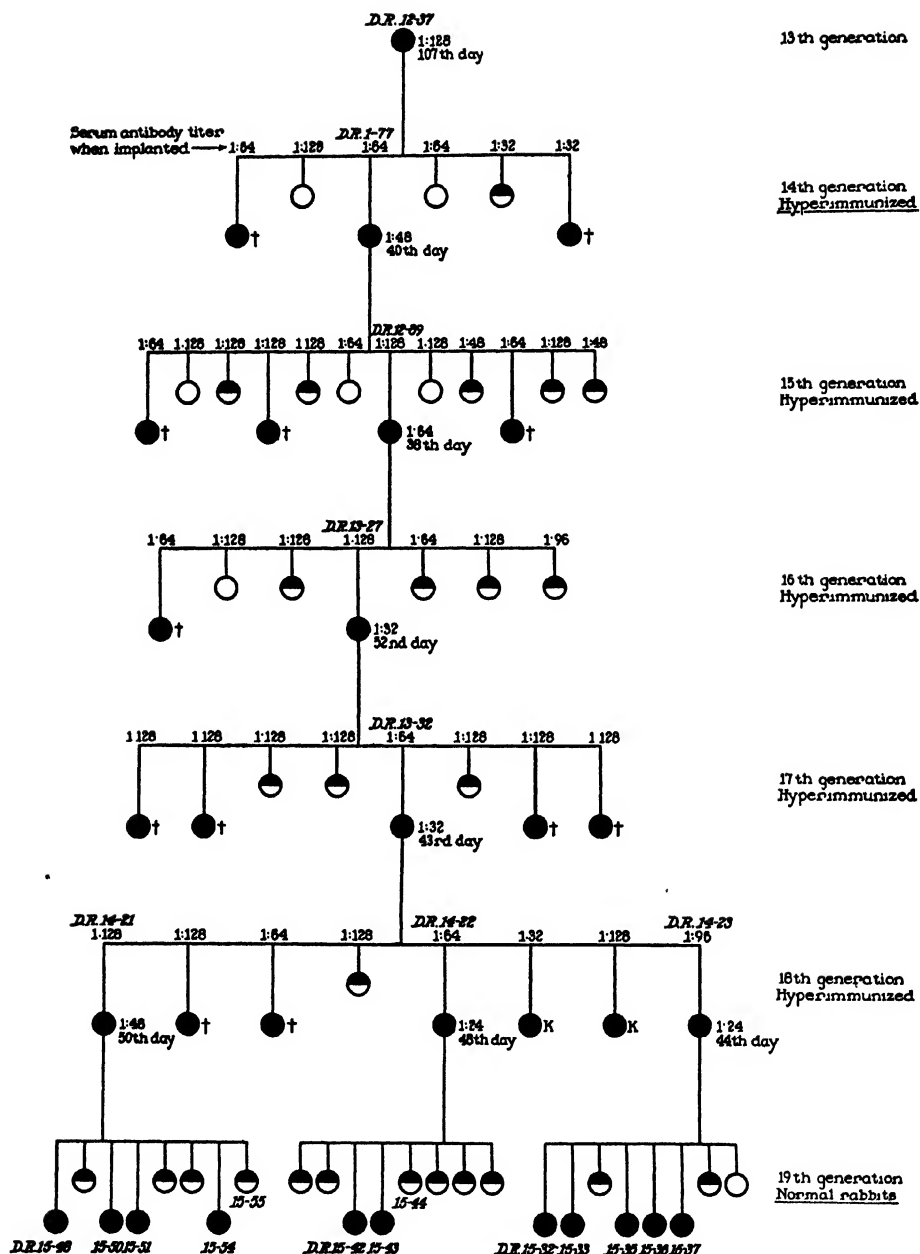


CHART 1. A summary of the transplantations. The black circles denote individual rabbits in which tumors grew progressively; half-white ones, animals in which regression occurred; white ones, rabbits in which no palpable nodules developed. The animals remain anonymous except for those providing material for transfer and the ones bled for serum in the final generation—see Table III. D. R. = domestic rabbit; K = killed; † = died from tumor. For further explanation see text.

the usual situations—in D. R. (domestic rabbit) 12-37, amongst others. Palpable nodules were present in three of the situations when the animal was examined on the 17th day, and in two more later on. By the 42nd day the five nodules range from 1.2 to 3.2 cm. in size. All enlarged greatly in the ensuing 8 weeks, at length reaching diameters of 7.5 to 10.0 cm., the animal during this time becoming thin and weak. The antiviral antibody titer of its blood as determined by complement fixation was found to be 1:128 on the 107th day, when it was killed and the tumors procured for transplantation. They were huge cysts of the sort already described (3), filled with thick, glairy, mucoid fluid in which floated yellowish gouts of necrotic material, and with walls 1 to 3 mm. thick, composed of pale-pink, close-texture tumor tissue. The healthiest portions of the neoplastic tissue were pressed through the monel metal sieve and suspended in a mixture of Tyrode and the animal's own serum (proportion 20:1)

First Transfer to Hyperimmunized Rabbits, 14th Generation.—The tumor suspension of D. R. 12-37, described above, was implanted into the six muscle situations of six hyperimmunized rabbits. At the time all bore four large confluent papillomatous masses, each about 6×8 cm. across and raised 0.4 to 0.8 cm., as result of inoculation 30 days before with W. R. (wild rabbit) 1-72 virus, 1:20. Intraperitoneal injections of 10 cc. of virus filtrate W. R. 1-68, 1:20, had been made on the 14th and 21st days and the serum antibody titers now ranged from 1:32 to 1:128. Progressively enlarging carcinomas developed in three of the implanted animals, early regression took place in one, and no palpable tumors developed in the other two (see chart).

Further Transfers in Hyperimmunized Animals.—By the 40th day after implantation the six growths of D. R. 1-77 (14th generation—hyperimmunized) had become cysts of characteristic sort, varying from 3.5 to 7.5 cm. in diameter. The rabbit was bled and the complement fixation titer found to be 1:48. It was killed and a suspension made by sieving the healthy portions of its tumors into Tyrode plus the animal's own serum (10:1). The suspension was then implanted into the leg muscles of twelve hyperimmunized rabbits (15th generation) all of which carried large confluent papillomas resulting from the inoculation of W. R. 1-28 virus, 1:10, 24 days before. The animals had been injected intraperitoneally with 10 cc. of virus filtrate W. R. 16-96, 1:10, on the 12th day, and with 10 cc. of virus filtrate W. R. 1-68, 1:10, on the 18th day, and they now had serum antibody titers ranging from 1:48 to 1:128. The implantations resulted in progressively enlarging tumors in four rabbits, regressing ones in five, and no palpable growths in three.

The details of the remaining transfers into hyperimmunized animals need not be given, for they do not differ significantly from those just furnished.

The Eventual Transfer to Normal Animals.—Tumors grew progressively in seven of the eight implanted rabbits of the 18th generation—the fifth hyperimmunized group. The growths of three (D. R. 14-21, 14-22, and 14-23) were implanted in normal rabbits, as indicated in the chart, each material into a different group of eight animals. For the final transfers sieved suspensions were made as usual, but plain Tyrode was used instead of Tyrode plus host serum, since it was deemed advisable to avoid the passive transfer of antibody as possibly affecting the outcome of tests of the blood of the new hosts. As the chart shows, the implantations from D. R. 14-21 grew progressively in half of the eight normal rabbits, regressing in the others; the D. R. 14-22 tumors gave progressively enlarging growths in two instances, regressing

ones in four, and no palpable nodules in two; while the D. R. 14-23 material provided progressively enlarging tumors in five rabbits, regressing growths in two, the eighth individual remaining negative. Details of the serum tests will be given further on.

General Findings

When viewed in the large, the results of the transfers, as summarized in Chart 1, leave no doubt that the V2 carcinoma can be readily propagated in animals hyperimmunized against the papilloma virus. It was maintained in this way in five successive tumor generations during nearly 8 months in all. Implantation of the growth into animals having very high titers of antiviral antibody often resulted in tumors that appeared promptly and grew progressively, frequently killing the hosts; but the outcome of the implantations varied much from one generation to the next and from individual to individual in the same generation. The question arises, therefore, whether the tumor grew as well in virus-immune animals as in comparable normal ones. Chart 1 goes far towards answering the question. The cancer flourished in many of the hyperimmunized hosts, its course comparing favorably with that in the tumor generations before hyperimmunization was undertaken (3), and when it was eventually returned to normal animals the number of "takes" and of progressively enlarging and regressing growths, though differing somewhat from one group of hosts to another, was on the whole no different from that obtaining in the last generation of hyperimmunized rabbits. To safeguard the tumor it had been propagated in normal animals as well as in the hyperimmunized ones throughout the period of experimentation. Hence the material for an additional comparison is available (Table I).

The passages in normal rabbits were made during the same months as those through the hyperimmunized but were not done simultaneously, and the materials used, though of the same tumor generations, came of necessity from different groups of animals. Furthermore, hashed tissue was implanted into the new normal hosts, and sieved suspensions into the hyperimmunized; and the animals of both groups were market bought, which is to say that none was pure bred. Even so, it becomes evident from Table I that the outcome of implantations of the V2 carcinoma, though varying somewhat as in the case of other propagated neoplasms, was not significantly different in the hyperimmunized and the normal rabbits. "Takes" developed in two-thirds or more of the implanted rabbits in all of the groups (Table I). Progressively enlarging tumors developed in from 28 to 87 per cent of the hyperimmunized animals and in from 17 to 54 per cent of the normals; while regression took place in from 13 to 56 per cent of the hyperimmunized rabbits and in from 33 to 83 per cent of the controls.

From the findings just given it is manifest that the V2 carcinoma grew as well in the hyperimmunized rabbits as in the normals; and this fact makes it seem altogether unlikely that the antibody titer of the hyperimmune rabbits at the time of implantation would have any influence on the outcome. That it had none can readily be seen from an inspection of Chart 1. In the 14th gen-

eration, for example, tumors enlarged progressively in three animals having antibody titers when implanted of 1:64, 1:64, and 1:32, respectively; no palpable nodules appeared in a rabbit with antibody titer of 1:64; and regression of the growths took place in two animals with titers of 1:32 and 1:128, respectively. So too in the 15th and the succeeding generations: the tumors grew, or failed to develop, or regressed irrespective of the antibody titer at time of implantation.

TABLE I

Outcome of Implantations of the V2 Carcinoma in Rabbits Hyperimmunized against the Papilloma Virus and in Normals

Generation	Number of rabbits implanted		Number developing palpable nodules		Number in which tumors grew progressively		Number in which tumors regressed	
	Hyperimmunized	Normal	Hyperimmunized	Normal	Hyperimmunized	Normal	Hyperimmunized	Normal
14th	6	11	4 (67%)	8 (73%)	3 (50%)	6 (54%)	1 (17%)	3 (33%)
15th	12	9	9 (75%)	8 (89%)	4 (33%)	3 (33%)	5 (42%)	5 (56%)
16th	7	12	6 (84%)	11 (92%)	2 (28%)	2 (17%)	4 (56%)	9 (75%)
17th	8	12	8 (100%)	12 (100%)	5 (62%)	2 (17%)	3 (38%)	10 (83%)
18th	8	8	8 (100%)	8 (100%)	7 (87%)	4 (50%)	1 (13%)	4 (50%)

Hybrid agouti rabbits procured from various dealers were used throughout.

The transplantations in corresponding hyperimmunized and normal generations were made during the same months but not simultaneously.

No distinction is made in the table between the animals in which regression was complete and the occasional ones in which it was partial and transitory, *i.e.*, individuals in which all of the tumors dwindled for a time and some disappeared but one or more later enlarged.

Attention may be called in passing to the fact that the antibody titers of the hyperimmunized rabbits that provided tumors for transplantation were lower when the animals were killed than at time of the implantations, 40 to 52 days before (Chart 1). The finding was not unexpected. For the high titers resulting from the injections of large quantities of virus intraperitoneally are not maintained, as previous experience had shown, and the antibody response was presumably at or near its height at the time the implantations were made. In many unimplanted rabbits immunized in other experiments the antibody titers fell during the 6 to 8 weeks following the intraperitoneal injections to less than one-half the maximum. Manifestly the virus present in the large papillomas growing on the bellies of the rabbits of the present experiments and in the V2 carcinomas proliferating in their leg muscles failed to keep the antibody

titer at its highest level. Such findings will not seem strange to students of the infectious diseases. Two possible reasons for them may be mentioned in the present case. The papillomas of domestic rabbits provide in general comparatively little antigenic stimulus, eliciting much lower titers of antiviral antibody than do growths of comparable size and duration in cottontails (9); and the V2 carcinoma, which eventually elicits very high titers of the antiviral antibody, does so only after the tumors have become large and cystic and have persisted for many weeks, as observations made recently with the collaboration of Dr. William F. Friedewald attest.

It is of interest to compare the course of the implanted V2 carcinomas with that of the cutaneous papillomas present on the same animal, for the reason that their relation to the host is very different. The cells of the V2 carcinoma come from another animal—the one in which this tumor first arose—whereas those of the papilloma are the animal's own, now infected with virus. Regression of the V2 carcinoma, like that of other transplanted cancers, would appear to be consequent upon an induced general resistance, directed against the foreign cells (10); whereas regression of the papilloma involves a resistance which is directed against such of the animal's own cells as have been rendered neoplastic by the virus (11). In neither case does the antiviral antibody have any influence on the fate of the tumor: growths of both sorts often dwindle away in rabbits having little or no antiviral antibody in their blood, and they frequently grow progressively in other animals having high serum antibody titers. Can it be that regression of the V2 carcinoma and that of the papilloma depend upon the same mechanism?

A summary is given in Table II of the course of the papillomas and carcinomas in the 41 hyperimmunized rabbits of Chart 1. The papillomas grew progressively in all of the rabbits except two, whereas in six rabbits no carcinomas appeared and in fourteen others they regressed after having attained a diameter of 1 cm. or more by the 16th day after implantation. In twelve of them the nodules had regressed completely by the 40th day or shortly thereafter; in the other two regression was partial and transitory, with dwindling of the nodules between the 16th and 30th days, complete disappearance of several of them, and later enlargement of one or two.

The V2 carcinoma grew progressively in the one rabbit in which the papillomas regressed entirely. This animal (D. R. 12-83, 15th generation—hyperimmunized) developed only scattered discrete papillomas as result of the inunction of the highly pathogenic W. R. 1-28 virus, 1:10, and these attained a height of 1 to 2 mm. on the 25th day; but had all regressed completely by the 38th day. The V2 carcinomas, implanted on the 25th day after virus inunction, grew vigorously and progressively from the start at every situation, becoming 1.5 to 2.4 cm. in diameter by the 38th day, that is to say, during the period when the surface papillomas were regressing. They continued to enlarge, formed huge cysts 4.0 to 6.0 cm. across, and brought about the animal's death on the 88th day (63rd day after the implantations).

From the data just given (Table II) it becomes evident that virus-induced papillomas of autochthonous origin and V2 carcinomas resulting from transplantation may grow or regress independently of one another in the same host.

The findings do not allow a definite conclusion as to whether regression of the two types of growths is brought about by similar mechanisms, but they provide a sound basis for doubting that the mechanism is identical in the two cases. Local conditions and other factors as well have an important influence upon the course of virus-induced papillomas (11), and similar influences may be responsible for the highly various outcome of implantations with the V2 carcinoma.

The passage of the V2 carcinoma through the hyperimmunized animals did not result in any perceivable alteration in the neoplasm. Autopsies were done as routine on all animals dying from the tumor, and representative blocks were taken for microscopic study. No differences could be made out between the growths in the hyperimmunized rabbits and those in the normals.

TABLE II

Fate of Autochthonous Virus-Induced Papillomas and Transplanted V2 Carcinomas in the Hyperimmunized Rabbits of Chart 1

	Number of rabbits
Papillomas and carcinomas grew progressively.....	20
Papillomas grew progressively—carcinomas failed to "take".....	6
Papillomas grew progressively—carcinomas regressed.....	13*
Papillomas dwindled (partial regression) and carcinomas regressed completely.	1
Papillomas regressed completely and carcinomas grew progressively.....	1
Total.....	41

* Partial regression in two.

Tests for Presence of Virus

To determine whether the virus had persisted in the V2 carcinoma during its transfer serially through hyperimmunized animals, the growths of three rabbits of the 18th generation (hyperimmunized) were transplanted into as many groups of normals (Chart 1). Later on tests were made for the antiviral antibody in the blood of the rabbits in which the tumors had grown progressively.

Table III summarizes the results of the serum tests. It will be seen that five of the rabbits with carcinomas derived from D. R. 14-23 all had huge growths when bled on the 61st day following implantation. Their sera contained much antiviral antibody, as manifested both by their capacity to react with the virus in the complement fixation test and by their ability to neutralize a potent suspension of it.¹ The tumors grew progressively in only two of the rabbits implanted with the growths of D. R. 14-22 and in only four of those implanted with the growths of D. R. 14-21. The antiviral antibody was present in quantity in the sera of all these, as the table shows. The sera

¹ The fact is conspicuous that the sera failed to neutralize the virus completely, though many had high titers of antiviral antibody as determined by complement fixation. The phenomenon has already been discussed in relation to the limitations of the neutralization test as a gauge of antibody titer (12).

TABLE III

Tests for Antiviral Antibody in the Blood of Rabbits Carrying V2 Carcinomas

19th Tumor Generation: Growth Implanted in Normal Animals after 5 Serial Transfers in Virus-Immune Rabbits

Source of serum		Im- planted	Diameter of growths (six situations)	Complement fixation tests†										Neutralization tests							
Normal rabbits implanted with V2 carcinoma				days	cm.	Serum dilution						19th day				43rd day					
From immune rabbits	No.					1:2	1:4	1:8	1:16	1:32	1:64	1:128	A	B	C	D	A	B	C	D	
D. R. 14-23	15-32	61	8-12-14-14-8-12	++++	++++	++++	++++	++++	++++	0	0	0	0	0	0	+	+	+	+		
	15-33	"	6-8-10-14-6-8	++++	++++	++++	++++	++++	++++	++++	+	+	+	+	+	+	+	+			
	15-35	"	5-7-9-14-8-4	++++	++++	++++	++++	++++	++++	++++	++++	+	+	+	+	+	+	+			
	15-36	"	8-8-8-11-9-10	++++	++++	++++	++++	++++	++++	0	0	0	0	0	0	0	0	0			
	15-37	"	10-9-12-14-9-8	++++	++++	++++	++++	++++	++++	++++	++++	+	+	+	+	+	+	+			
D. R. 14-22	15-42	57	5-8-8-8-4-5	++++	++++	++++	++++	++++	0	0	0	0	0	0	0	+	+	+			
	15-43	"	6-8-10-10-4-10	++++	++++	++++	++++	++++	++++	++++	+	+	+	+	+	+	+	+			
	15-44	"	N-N-N-3.5-N-N	0	0	0	0	0	0	0	0	+	+	+	+	+	+	+			
	15-48	55	10-7-10-10-6-6	++++	++++	++++	++++	++++	++++	++++	++++	+	+	+	+	+	+	+			
D. R. 14-21	15-50	"	5-4-7-8-4-2	++++	++++	++++	++++	++++	++++	++++	++++	+	+	+	+	+	+	+			
	15-51	"	8-7-10-10-9-7	++++	++++	++++	++++	++++	++++	++++	++++	+	+	+	+	+	+	+			
	15-54	"	4-5-6-8-8-6	++++	++++	++++	++++	++++	++++	0	0	0	0	0	0	0	0	0			
	15-55	"	Regressed-neg.	±	0	0	0	0	0	0	0	0	0	0	0	+	+	+			
	15-56			0	0	0	0	0	0	0	0	+	+	+	+	+	+	+			
Normal con- trols—not implanted	15-57			0	0	0	0	0	0	0	+	+	+	+	+	+	+	+			
	15-58			0	0	0	0	0	0	0	+	+	+	+	+	+	+	+			
Saline control				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			

† 2 units of complement in all tubes.

Antigen, P, 1:120.

None of the sera was anticomplementary when tested concurrently in double volume, nor was the antigen.

† Equal parts serum and 1 per cent virus filtrate P, incubated 2 hours at 37°C.

of the three normal controls exhibited no capacity to fix complement and were devoid of ability to neutralize the virus.

Special attention should be called to the findings with the sera of D. R. 15-44 and 15-55, rabbits implanted respectively with the growths of D. R. 14-22 and 14-21. In D. R. 15-44, nodules 1.0 to 2.5 cm. in diameter had appeared at all six situations by the 17th day following implantation. They measured from 1.8 to 4.0 cm. on the 29th day, but during the ensuing 2 weeks all abruptly vanished except the largest, which dwindled to a softish nodule 1.2 cm. across. Later on this gradually enlarged and it had become a thin-walled cyst 3.5 cm. across at the time of bleeding. The serum of this rabbit had no detectable capacity to fix complement, but it possessed the power to neutralize partially a 1 per cent virus filtrate (Table III). The growths of D. R. 15-55 were indolent. Palpable nodules 0.4 and 1.2 cm. across were present in only two of the six implanted situations on the 15th day. Five nodules measuring 0.4 to 2.0 cm. had appeared by the 27th day, but these dwindled during the next 2 weeks and all had disappeared when the animal was bled on the 55th day. The animal's serum had only the slightest complement-fixing capacity, though it neutralized a considerable proportion of the test virus as the table shows. The results contrast sharply with those got with the sera of rabbits in which the tumors had grown big. They conform to a general rule, namely, that rabbits in which the V2 carcinoma fails to grow, and those in which it regresses before it has got large, fail as a rule to develop the antiviral antibody in quantity (3).

The telltale antibody in the blood of all of the rabbits in which the cancer grew (Table III) makes plain the fact that the virus was still associated with the V2 carcinoma despite the propagation of the tumor during a period of nearly 8 months in hosts hyperimmunized against it. The fact that the antibody appeared in as high titer as previously when the growth was returned to normal animals indicates that the tumor tissue contained the usual amount of antigenic material, namely, masked or altered virus. The conclusion would seem warranted that the latter had increased to the usual extent as the V2 carcinoma grew in the hyperimmune hosts.

COMMENT

It is generally recognized now that viruses are protected from the action of specific antiviral antibodies so long as they remain associated with living susceptible cells.² No exception to the rule has been discerned: even the necro-

² It is of theoretical interest to speculate upon the mechanism whereby living cells protect viruses from the action of circulating antiviral antibodies. Perhaps simplest is the assumption that viruses live within cells and have no contact with antibodies, these being presumably kept away by the semipermeable protoplasm. But it is conceivable that specific antiviral antibodies might still be ineffective even if they reached the vicinity of the virus. This might be the case, for example, if, in the living cell, there were a special association between the cell constituents and that part of the virus to which the antibody would become attached in the absence of such an association.

tizing viruses are protected until they kill their cell hosts and thus expose themselves to the action of the antibodies they have elicited (13). The principle finds exquisite illustration in the case of the papilloma virus, which, inducing neoplastic proliferation, continues to exert its effects and to increase in amount in association with cells that are nourished by blood which would promptly render the virus inactive in the lack of cell protection (14).

The same state of affairs exists in the V2 carcinoma, and under conditions even more extreme; for the virus continues to increase even when the growth is propagated in hosts previously immunized against it, as the foregoing experiments have shown. The outcome is notably different, however, in the case of certain of the extraneous viruses that sometimes ride along as passengers in transplanted cancers. For, as already mentioned, virus III and vaccine virus—the only ones tested thus far in this relation—are eliminated if the tumors they ride upon are propagated in hosts previously immunized against them (6, 7). It is conceivable that the passenger viruses disappear because not sufficiently protected, but this is not necessarily so. Both of the aforementioned viruses can survive in tumors after the hosts become immune to them (16),—findings which show clearly that tumor cells are capable of protecting some passenger viruses, at least against ordinary amounts of antibody.

The disabilities inherent in the characters and effects of passenger viruses will sufficiently account for their disappearance from tumors propagated in virus-immune hosts. Many of them sooner or later kill the cells susceptible to them; and under ordinary circumstances they rely upon passage to new cells for survival and increase. None is a neoplastic virus, going along with cells that proliferate continually as result of its action. Their disappearance from tumors propagated in virus-immune hosts could be explained by supposing that they infect relatively few of the tumor cells at any one time, that they seriously interfere with cellular proliferation, and that they maintain their association with the tumors chiefly by virtue of their ability to pass from dead or dying cells to living susceptible ones, instead of going along as the cells divide. For under such circumstances the neutralizing antibody already present in the new host would inactivate any virus liberated from the infected cells and preclude the infection of susceptible ones, and the uninfected cells would sooner or later outgrow those hampered by the parasite.

This does not mean that all passenger viruses would necessarily be eliminated by similar means. Certain extrinsic viruses can adapt themselves notably well to tumor cells, as witness the persistence of fowl pest and lymphogranuloma viruses in tumors apparently unaffected by them (17). But it is one thing to go along with a tumor and another to be its cause or to modify its neoplastic character. None of the passenger viruses thus far studied has either of these effects though some induce the formation of inclusion bodies in tumor cells and many cause cellular necrosis and thus deter tumor growth (4). In sum, how-

ever, the passenger viruses are all mere riders. The papilloma virus on the other hand is not only responsible for the causation and continued proliferation of tumors, but it has proved capable of rendering malignant many benign tar tumors of the rabbit and of hastening the growth and modifying the character of others, both malignant and benign, when brought into contact with them experimentally (15). In previous papers facts have been presented which attest to the continued neoplastic activities of the virus in partnership with the V2 carcinoma cells (3, 15).

SUMMARY

The V2 carcinoma—a transplanted rabbit cancer derived originally from a virus-induced papilloma and carrying in masked or altered form the virus primarily responsible for it—was propagated in five successive groups of animals all previously hyperimmunized against the papilloma virus. The cancer grew as well in the hyperimmunized hosts as in normal animals implanted during the same months; and serological tests, made when the tumor was eventually returned to ordinary hosts, proved that the virus was still associated with the carcinoma cells: it had increased to the usual extent as the tumor grew in the hyperimmune animals.

The continued increase of the neoplastic virus during propagation of the V2 carcinoma in hyperimmunized hosts contrasts sharply with the elimination of certain extraneous passenger viruses when the tumors they ride upon are grown in hosts previously immunized against them. The facts as a whole would seem to warrant a distinction between the enduring partnership of a neoplastic virus and carcinoma cells on the one hand and the casual association of passenger viruses with tumor cells on the other.

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CELL STATE AS AFFECTING SUSCEPTIBILITY TO A VIRUS

ENHANCED EFFECTIVENESS OF THE RABBIT PAPILLOMA VIRUS ON HYPERPLASTIC EPIDERMIS

By WILLIAM F. FRIEDEWALD, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 2 AND 3

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The effects of some viruses are known to be altered when they act upon cells which have been previously rendered abnormal. The virus of herpes simplex induces lesions resembling those of herpes zoster in rabbit skin altered by tar (1), and ultraviolet irradiation of rabbit skin modifies the local susceptibility to inoculation with vaccine virus (2). The Shope papilloma virus (3) elicits carcinomas forthwith, as well as various papillomas of unusual sort, when it is brought into association with the epidermis of domestic rabbits, which has been tarred for some weeks (4). The experiments reported in the present paper were undertaken to learn whether preliminary alterations of rabbit epidermis would provide a more favorable soil for the demonstration of the papilloma virus. Often no virus can be got from the papillomas of domestic rabbits directly produced therewith though its presence in the growths is readily demonstrable serologically (5, 6) as also in the cancers deriving from them (7).

Material and Methods

Papilloma virus was obtained from the naturally occurring growths of cottontail rabbits which had been preserved in 50 per cent glycerin-Locke's solution at about 4°C. Weighed portions of the tissue were passed through several changes of saline, ground with sand, and suspended in 10 to 20 volumes of 0.9 per cent saline. The crude extracts thus obtained were spun in an angle-head centrifuge at about 3500 R.P.M. for 5 minutes and the supernatant fluids were again spun at about 4500 R.P.M. for 20 minutes. They were now clear amber and were usually highly infectious as such and after filtration through Berkefeld V candles. For test they were inoculated intradermally or rubbed into the skins of adult domestic rabbits of the gray-brown (agouti) breed or cottontail rabbits (genus *Sylvilagus*) after scarification with sandpaper, according to a method already described (5, 6). The character of the papillomas arising was recorded at frequent intervals from the 8th day to about the 42nd day after inoculation according to a standard scale: ++++ = confluent papillomatosis, +++ = semiconfluent papillomatosis, ++ = many discrete papillomas, + = 5 to 15 papillomas, ± = 2, 3, or 4 papillomas, ± = 1 papilloma, 0 = negative.

To conserve space, the readings of only a few days are given in the tables. They are representative of the findings as a whole.

Influence of Carcinogenic Agents to Alter Skin Susceptibility

In a first experiment the results of rubbing a suspension of papilloma virus into scarified skin were compared with those consequent on infiltrating tarred rabbit ears with the suspension by way of a marginal vein.

Experiment 1.—The ears of fourteen normal cottontail rabbits were tarred over the inner and outer surfaces on three occasions at intervals of 3 or 4 days. This amount of tarring caused the ears to become acutely inflamed, thickened, and moist, but elicited no tumors. A strong rubber band was placed about the base of the ear, to stop the circulation, and 5 cc. of a virus filtrate (W.R. 1-28) diluted from 1:500 to 1:480,000,—in terms of the papilloma material originally extracted,—was then slowly injected into a marginal vein of the ear. The rubber bands were removed 5 minutes after injection. The ears of each rabbit received different dilutions of the virus. 0.2 cc. of the same dilutions was then rubbed into scarified areas of normal skin on the abdomens of the same rabbits.

The results can be briefly summarized. Six of the rabbits proved resistant to infection, only a few growths arising from either the tarred or the normal epidermis. Eight rabbits were susceptible and in every one many more papillomas developed from the tarred epidermis than from the normal skin inoculated with the same dilution of virus. The ratio of the number of papillomas on the tarred skin to that on the normal skin was as high as 135 or more to 1. Furthermore papillomas were elicited in the tarred ears by a considerably higher dilution of virus. In one instance a dilution of 1:80,000 elicited papillomas on the tarred epidermis, yet 1:500 failed to do so on the untreated skin. In five animals papillomas appeared on the tarred ears and none on the scarified skin in response to the same dilution of virus. All of the growths were benign papillomas.

The findings indicate that rabbit skin to which tar has been applied a few times is more susceptible to infection with the papilloma virus than normal scarified skin. The objections can be raised, however, that the total amount of virus introduced into the tarred ears was greater than that rubbed into the skin of the abdomen and that neither the skin situations nor the methods of inoculation were comparable. To control these variables an experiment was done next in which virus was rubbed into areas of skin, some of which had been tar-treated while others had been left untreated prior to scarification. As enlarging the test, other areas which had been treated previously with methylcholanthrene or benzpyrene were inoculated with virus.

Experiment 2.—The hair was clipped from twelve rectangular areas of skin measuring about 3×5 cm. on the abdomens of four domestic rabbits. Small hairy strips were left between the areas which were in three anteroposterior rows of four areas each. One area of each row was painted with a carcinogenic tar, another with 0.3

per cent methylcholanthrene in benzene, and a third patch with 0.3 per cent benzpyrene in benzene.¹ Small camel's hair brushes were used to apply the agents. The remaining area of each row served as control. The situations of the control and treated areas were varied from animal to animal. After each treatment they were covered individually with sterile gauze pads, moored with adhesive to prevent spread of the reagents, after which a gauze pad and many-tailed binder were put over all. The skin treatments were repeated twice per week for 2 weeks. They elicited no tumors.

Two days after the last treatments the patches were greatly changed. Those that had been tarred showed on stripping an acutely inflamed and thickened epidermis covered with a moist yellowish-brown scurf. The methylcholanthrene-treated skin was also inflamed and greatly thickened, but less so, and was covered with a dry brown scurf. The benzene-treated skin had a similar appearance. To learn the nature of the changes a narrow slice of a normal area and of each of the treated patches was taken under ether anesthesia from each rabbit, fixed in acid Zenker solution, and stained with eosin and methylene blue.

Immediately after the biopsies the areas were scarified with sandpaper and a 0.5 per cent virus extract (W.R. 1-28) was rubbed into one row of the normal and treated skin areas, and another similarly prepared extract (W.R. 11-69) into another row. The treated areas proved more difficult to scarify than the normal skin, the tar-treated areas especially, because of the moist adherent scurf. Immediately after inoculation the patches were dried in a blast of warm air and bandaged as before.

The results of the experiment are shown in Table I. The findings with the two virus extracts can be considered together. On the 10th day after virus inoculation, discrete papillomas had appeared on most of the methylcholanthrene-treated skin areas and on a few of the benzpyrene- and tar-treated areas. No growths were visible on the normal skin at this time. By the 15th day the papillomas on the treated skin areas had greatly increased in number and had enlarged rapidly, many of them now being semiconfluent papillomatous masses. The normal skin areas, on the other hand, showed relatively few papillomas and they were small and discrete. On the 20th day most of the treated skin areas showed large, vigorous, confluent or semiconfluent papillomatous masses up to 1.0 cm. high, while the growths on the normal skin were still discrete, in some instances just appearing, and only occasionally were as much as 0.3 cm. high. The papillomas on the treated patches were in many instances dark gray while those on the healed normal skin of the same animal were pink or light gray. All were ordinary virus-induced papillomas.

The experiment shows (Table I) that a few preliminary applications to rabbit skin of tar, benzpyrene, or methylcholanthrene greatly enhance the susceptibility of the skin to papilloma virus infection. Growths appeared earlier in the treated skin, were more numerous, tended to be more pigmented,

¹ The tar came from the Ostergasfabrik of Amsterdam, and was the generous gift of Dr. Karl Landsteiner. It has been employed in much work in this laboratory. The methylcholanthrene was obtained from the Eastman Kodak Company and the benzpyrene from Hoffmann La Roche, Inc.

and grew more rapidly, forming large papillomatous masses at a time when the growths in the normal skin were just appearing or were still small and discrete. Similar findings were obtained in another experiment when virus was inoculated into normal and methylcholanthrene-treated skin by means of a tattoo machine (Figs. 6 and 7).

TABLE I

Susceptibility to the Papilloma Virus of Normal Skin and Skin Treated with Carcinogenic Agents

Papilloma virus extract	Agent applied to skin before virus inoculation*	Pathogenicity tests											
		10th day				15th day				20th day			
		a	b	c	d	a	b	c	d	a	b	c	d
Test rabbits.....													
No.													
W.R. 1-28 (0.5 per cent)	Normal skin	0	0	0	0	+	±	±	+±	++	++	+±	++±
	Tar	0	0	0	+	+±	+++	+	+++	++++±	+++++	++++	+++++
	Benzyrene	+	0	0	±	++±	+++	++	+++±	+++	+++±	++++	+++++
	Methylcholanthrene	+±	±	±	+±	+++	+++	+++	+++±	++++	++++	++++	++++
W.R. 11-69 (0.5 per cent)	Normal skin	0	0	0	0	+	±	±	+±	++	+±	+	++
	Tar	0	0	0	0	±	+++	±	+++	++	++++	++	++++±
	Benzyrene	0	0	0	±	++	+++	+	+++	+++±	++++	+++	+++±
	Methylcholanthrene	+	±	0	±	+++	+++	++	+++	++++	++++	+++	+++±

* Benzyrene and methylcholanthrene (0.3 per cent in benzene) and tar applied twice per week for 2 weeks. Virus inoculated 2 days after last treatments.

++++ = confluent papillomatosis.

+++ = semiconfluent papillomatosis.

++ = many discrete papillomas.

+

± = 2, 3, or 4 papillomas.

± = one papilloma.

0 = negative.

Effect of Non-Carcinogenic Agents to Alter Skin Susceptibility

The agents used in the preceding experiment were potent carcinogens, but were employed for so brief a period that they elicited no tumors. A test was next done to find whether non-carcinogenic agents, inflammatory for rabbit skin, would have the same effect.

Experiment 3.—Twelve skin areas on the abdomens of four domestic rabbits were clipped as in Experiment 2. Three on each animal were painted with benzene as such and three others with 0.3 per cent methylcholanthrene in benzene three times per week for 2 weeks. Three more areas were exposed to 1500 r of x-ray irradiation 48 hours before virus inoculation. The rabbits were protected by flexible lead foil during irradiation, leaving only the areas exposed, and the rays came from a single tube run at 5 milliamperes and at a peak voltage of 135 kilovolts, without filtration.

The distance from tube to skin was 50 cm. The three remaining skin areas served as controls. A representative piece of the normal and treated skin areas was taken for microscopic study 2 days after the last treatments. The methylcholanthrene-treated skin showed the same gross changes described in Experiment 2. A 5 per cent virus filtrate (W.R. 1-30) was then rubbed into one area of each sort after scarification and into another after dilution of the filtrate to 0.1 per cent. The inoculations were made immediately after the biopsies.

Table II shows the results of the experiment. On the 12th day after virus inoculation the benzene- and methylcholanthrene-treated patches inoculated with 0.1 per cent filtrate showed discrete papillomas in three of the four test rabbits. No growths could be seen on the normal or x-rayed skin areas at this time. All of the areas inoculated with 5 per cent filtrate showed papillomas, the growths on the methylcholanthrene-treated areas being large, semiconfluent masses in contrast to the few small discrete growths just appearing on the normal and x-rayed patches. Many more discrete papillomas were present on the benzene-treated areas than on the normal skin, but they were fewer in number and smaller than on the skin areas which had received methylcholanthrene. By the 35th day the papillomas on the benzene- and methylcholanthrene-treated areas inoculated with the dilute filtrate had developed into confluent and semiconfluent papillomatous masses, whereas the growths on the normal and x-rayed skin areas produced with the same inoculum were still small and discrete though increasing in number. The skin areas inoculated with 5 per cent filtrate all showed confluent papillomas, but those on the normal and x-rayed patches were low mounds, up to 0.6 cm. high, whereas on the benzene-treated areas they were jagged peaks up to 1.0 cm. high and on the skin treated with methylcholanthrene they were up to 1.6 cm. high. The last mentioned growths were deeply pigmented, while the others were pink or merely streaked with gray. Microscopic sections of the growths showed them all to be ordinary papillomas.

To extend the findings another experiment was done. This time the skin areas were treated with ultraviolet light, methylcholanthrene, and a mixture of turpentine and acetone.

Experiment 4.—A mixture of turpentine and acetone in equal parts,—which had proved non-carcinogenic (8),—was applied to three clipped areas on the abdomens of four domestic rabbits five times at 2 day intervals and 0.3 per cent methylcholanthrene in benzene in like manner to another three. Three other areas were exposed to a quartz mercury vapor lamp² for 40 minutes at a distance of 25 cm. 48 hours before virus inoculation. This caused a marked erythema. Three untreated skin areas served as controls. The turpentine and acetone-treated skin became acutely inflamed, thickened and hyperkeratotic, as greatly changed in the gross as the methylcholanthrene-treated skin. A virus filtrate (W.R. 1-70) in concentrations of 0.1 per cent and 5 per cent was rubbed into two rows of the areas after scarification.

The results are shown in Table III. It will be seen that the papillomas on the methylcholanthrene- and turpentine and acetone-treated areas arose earlier and in

¹ Alpine sun lamp, Hanovia Chemical and Manufacturing Company.

TABLE II

Susceptibility of Normal Skin and Skin Treated with X-Rays, Benzene, and Methylcholanthrene

Dilution of virus filtrate W.R. 1-30		Skin treatment before virus inoculation*	Pathogenicity tests										
			12th day				18th day				35th day		
Test rabbits			a	b	c	d	a	b	c	d	a	b	c
per cent													
	0.1	Normal skin	0	0	0	0	±	±	+	±	++	+±	++
		X-rays	0	0	0	0	±	+	0	0	+±	++±	+±
		Benzene	+	+	0	+	+±	++	++	++	++++±	++++±	++++
Methylcholanthrene		±	+	0	±	+±	++	++±	+±	++++±	++++±	+++++	
5	Normal skin	±	+	±	±	++	+++	++±	++±	++++	++++	++++	
	X-rays	±	+	±	±	++	+++	+++	+±	++++±	++++	++++	
	Benzene	++	++±	++	++	++++±	++++	++++±	++++±	++++	++++	++++	
	Methylcholanthrene	+++	++++±	+++	++++±	++++	++++	++++	++++	++++	++++	++++	

* Benzene applied to skin areas three times per week for 2 weeks before virus inoculation.

0.3 per cent methylcholanthrene in benzene applied three times per week for 2 weeks before virus inoculation.

X-ray irradiation, 1500 r.

Virus inoculated 2 days after last treatments.

TABLE III

Susceptibility of Normal Skin and Skin Treated with Ultraviolet Light, Turpentine and Acetone, and Methylcholanthrene

Dilution of virus filtrate W.R. 1-70		Skin treatment before virus inoculation*	Pathogenicity tests											
			11th day				18th day				35th day			
Test rabbits.....			a	b	c	d	a	b	c	d	a	b	c	d
per cent	0.1	Normal skin	0	0	0	0	±	±±	+	±±	++	±±±±	±±±	±±±±
		Ultraviolet light	0	0	0	0	±±	±±	+	+	±±±	±±±±	±±±	±±±
		Turpentine-acetone	±	+	0	±	±±±	±±±±	++	±±±	±±±±	±±±±	±±±±	±±±±
		Methylcholanthrene	0	±	+	0	±±±	±±±	±±±	±±±	±±±±	±±±±	±±±±	±±±±
5		Normal skin	0	++	+	+	±±±	±±±±	±±±	±±±±	±±±±	±±±±	±±±±	±±±±
		Ultraviolet light	+	±±±±	++	++	±±±±	±±±±	±±±	±±±±	±±±±	±±±±	±±±±	±±±±
		Turpentine-acetone	±±	±±±	±±±	±±±	±±±±	±±±±	±±±±	±±±±	±±±±	±±±±	±±±±	±±±±
		Methylcholanthrene	±±	±±±	±±±±	±±±	±±±±	±±±±	±±±±	±±±±	±±±±	±±±±	±±±±	±±±±

* Ultraviolet light, 40 minutes irradiation from a carbon lamp 24 hours before virus inoculation.

Turpentine and acetone in equal parts applied to skin five times at 2 day intervals.

0.3 per cent methylcholanthrene in benzene applied to skin five times at 2 day intervals.

greater numbers than on those which had been normal. There was no significant difference between the growths on the treated areas and they all soon became large, confluent papillomatous masses. The papillomas arising on the areas which had been exposed to ultraviolet light were like those on the normal skin in number and size.

The results of Experiments 2, 3, and 4 (Tables I, II, and III) show that a variety of agents, some carcinogenic, others not, will render the skin abnormally susceptible to virus infection. A mixture of turpentine and acetone was as effective in this respect as was tar or methylcholanthrene. These findings have been confirmed in many subsequent tests in which the procedures have been utilized for various purposes. Mere acute inflammation produced by ultraviolet light did not render the skin more susceptible, nor did the Roentgen rays.

Optimal Preparation of the Skin for Virus Infection

Steps were now taken to determine the number of applications of methylcholanthrene or of turpentine and acetone which renders the skin most susceptible to the papilloma virus.

Experiment 5.—0.3 per cent methylcholanthrene in benzene was painted onto a skin area of each of four domestic rabbits at 2 day intervals for a total of six times, and another comparable skin area was simultaneously treated with a mixture of turpentine and acetone. Two other skin areas of the same animals were treated with the agents three times at 2 day intervals, while another two areas received but a single application. The schedule was so arranged that the last application was made 24 hours prior to virus inoculation. Two skin areas on each rabbit were left untreated. As usual the situation of the treated areas was varied. A single application of methylcholanthrene caused only a reddening of the skin after 24 hours. After three applications, however, the skin was acutely inflamed, thickened, and covered with a branny scurf. Six treatments caused even greater changes with marked thickening and much scurf, some of which could be flaked away. Turpentine and acetone caused similar changes, but the skin was slightly less thickened, although more inflamed. Biopsy specimens were taken from all of the areas. Immediately afterward the areas were scarified and a 0.5 per cent virus extract (W.R. 1-28) was rubbed into them.

The results are summarized in Table IV. It will be seen that a single application of methylcholanthrene or turpentine and acetone did not alter the susceptibility of the skin to papilloma virus infection; the incubation period and the number and size of growths were about the same on the normal and treated skin areas. Those treated three times, however, showed numerous papillomas before any could be seen on the normal areas and they had become large, confluent masses, up to 1.6 cm. high, at a time when growths on the normal skin were still discrete or semiconfluent and no more than 0.4 cm. high at most. Six applications of the agents rendered the skin only slightly more susceptible to virus infection than did three applications, in spite of the fact that it appeared much more changed, and histologically was really so, as will be shown further on.

Six applications of methylcholanthrene or turpentine and acetone at 2 day intervals are about the maximum that the skin of the rabbit's abdomen will bear without becoming macerated.³ It will withstand many applications of methylcholanthrene, however, if applied at 3 to 4 day intervals. Further experiments have shown that many treatments with these agents fail to render the epidermis more susceptible than do three to six applications at 2 day intervals.

TABLE IV
Susceptibility of Skin Prepared for Various Lengths of Time

Agent applied to skin before virus inoculation*	No. of applications	Pathogenicity tests†											
		12th day				16th day				24th day			
		a	b	c	d	a	b	c	d	a	b	c	d
Test rabbits,....													
Normal skin		0	0	0	0	+	±	±	++	+++	+++	++	+++
Turpentine-acetone	One	0	0	0	0	++	+	+	+	++++±	+++±	++	+++
	Three	±	±	±	++	+++	+++	+++	+++	+++++	+++++	+++++	+++++
	Six	+	+++	+++	+	+++	++++±	++++±	+++	+++++	+++++	+++++	+++++
Methylcholanthrene	One	0	0	0	0	+	±	±	±	+++	++	++	+++
	Three	±	±	+	++	++	+++	+++±	+++	+++++	+++++	+++++	+++++
	Six	±	++	+++	++	+++	+++	+++±	+++	+++++	+++++	+++++	+++++

* Turpentine and acetone in equal parts.

Methylcholanthrene, 0.3 per cent in benzene.

† Virus extract, W.R. 1-28, 0.5 per cent, rubbed into scarified skin 24 hours after preparation.

Duration of the Abnormal Susceptibility

Does the increased susceptibility of methylcholanthrene-treated skin to papilloma virus infection persist when applications of methylcholanthrene are discontinued? To answer this question skin areas were treated with methylcholanthrene in the next experiment and then inoculated with papilloma virus at various intervals from 1 day to 1 month after completion of the treatments.

Experiment 6.—Five areas on each of five rabbits were painted with 0.3 per cent methylcholanthrene in benzene at intervals of 2 days for a total of four times. The treatment of each skin area was begun on a different date so that from 24 hours to 4

³ The agents mentioned induce a profuse growth of hair. It has been found that removal of this with clippers prior to each application provides a better prepared epidermis which can be readily scarified.

weeks had elapsed on the day when they were all inoculated with 0.1 per cent virus filtrate (W.R. E). Just prior to inoculation biopsies were made as usual.

Table V shows the results of the Experiment. As noted in the preceding tests, papillomas appeared earlier and in greater number on the methylcholanthrene-treated skin inoculated 24 hours after completion of the treatments than on scarified normal skin. The areas inoculated with virus 1 week afterwards showed slightly fewer papillomas in four of the five test rabbits but in one instance there was no difference. The skin inoculated 3 weeks after discontinuation of the methylcholanthrene applications showed more growths than the normal skin in two instances only, and the patches inoculated 4 weeks after the applications had been stopped yielded the same results as the normal

TABLE V
Duration of the Increased Susceptibility of Methylcholanthrene-Treated Skin

Time from skin treatment* to virus inoculation†	Pathogenicity tests														
	14th day					16th day					21st day				
	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e
Test rabbits.															
1 day	+++	+++	+++	+++	±±	+++	+++	+++	+++	±±±	+++	+++	+++	+++	±±±±
1 wk.	±	±±	±±	±±±	±±	+++	+++	+++	+++	±±	+++	+++	+++±	+++±	+++
2 wks.	±	+	0	++	+	+++	+	±	±±±	±±	++	±±	±±	+++	±±±
3 wks.	0	0	0	±±	±	±	±	±	++	±±	+	±±	+	++	±±±
4 wks.	0	0	0	0	0	+	±	±	±±	±	+	+	±±	++	±±
Normal skin	0	0	0	0	0	+	±	±	+	±	++	+	±±	++	+

* 0.3 per cent methylcholanthrene in benzene applied to skin four times at 2 day intervals.

† Virus filtrate, W.R. E, 1:1000.

skin. It is thus apparent that the increased reactivity of methylcholanthrene-treated skin to papilloma virus infection is transitory and is lost within 2 to 4 weeks after the treatments are stopped.

Comparative Titrations of the Virus on Normal and Altered Skin

The increased number, shortened incubation period, and the rapidity with which the papillomas enlarged when virus was inoculated into properly prepared skin pointed to a markedly increased effectiveness of the virus. Experiments were next undertaken to learn whether it would be infectious in dilutions which yield no growths under ordinary circumstances. The virus is rarely infectious on the scarified normal skin of domestic rabbits in dilutions beyond 1:100,000 (in terms of weight of papilloma tissue extracted) (6).

Experiment 7.—Six squares were clipped on one side of the belly of four rabbits, and painted with a mixture of turpentine and acetone five times at 2 day intervals. They

showed the usual alterations. Six corresponding squares on the other side were not treated. All were scarified and a papilloma virus extract (W.R. 1-28) in dilutions of from 1:10,000 to 1:10,000,000 was rubbed into corresponding normal and treated squares. The inoculations were made 24 hours after the last of the turpentine-acetone applications.

It will be seen (Table VI) that by the 16th day after inoculation all of the treated areas which had received the 1:50,000 dilution of virus showed papillomas and the 1:100,000 had also caused them in one animal. No growths whatever had appeared at this time on the control squares. Later the control areas showed papillomas in every case as result of the 1:50,000 dilution, and the 1:100,000 dilution also caused them in the more susceptible animals. But

TABLE VI

Titration of a Virus Preparation in Normal and in Turpentine and Acetone-Treated Skin

Dilution of virus extract W.R. 1-28	Pathogenicity tests															
	16th day								42nd day							
	Normal skin				Turpentine and acetone-treated skin*				Normal skin				Turpentine and acetone-treated skin*			
	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
Test rabbits																
1:10,000	0	0	0	0	+	+	±	±	++++	++++	++++	±	++++	++++	++++	++++
1:50,000	0	0	0	0	±	±	±	±	+	+++	±	±	+++	+++	+++	+++
1:100,000	0	0	0	0	0	±	0	0	±	++	±	0	+++	+++	++	+
1:500,000	0	0	0	0	0	0	0	0	0	0	0	0	+	++	++	±
1:1,000,000	0	0	0	0	0	0	0	0	0	0	0	0	±	+	±	0
1:10,000,000	0	0	0	0	0	0	0	0	0	0	0	0	0	±	0	0

* Turpentine and acetone in equal parts applied three times per week for a total of five times before virus inoculation.

the virus had proved greatly more effective on the treated areas. At 1:100,000 it regularly produced papillomas, as did the 1:500,000 dilution, while in three of the four animals the 1:1,000,000 dilution proved effective, and in one individual the virus extract yielded papillomas when diluted 10,000,000 times.

The experiment shows that not only was the number of papillomas increased and the incubation period shortened by the use of treated skin but the titer of the virus was much stepped up—from 10- to 100-fold. Another, similar experiment, using methylcholanthrene-treated skin and another virus extract, yielded practically identical results. In a subsequent paper many instances will be given, incidentally to other work, of the enhanced susceptibility of treated skin.

Effect of Skin Alterations before and after Virus Inoculation

It seemed possible that methylcholanthrene or turpentine and acetone applications to the epidermis after virus inoculation might have the same

effect as preliminary treatment. An experiment was done to test the possibility.

Experiment 8.—Nine areas in three parallel rows were clipped on the abdomens of four rabbits. Those of one row were painted with 0.3 per cent methylcholanthrene in benzene at 2 day intervals for a total of four times, with result in the usual changes. The other six patches were not treated. Two days after the last treatment all were scarified and inoculated with a virus extract (W.R. 2-95), in dilutions of 1:50,000,

TABLE VII

Infectivity of Virus in Skin Treated with Methylcholanthrene before and after Inoculation

Skin treatment	Dilution of virus extract W.R. 2-95 used for inoculation	Pathogenicity tests											
		18th day				28th day				35th day			
Test rabbits		a	b	c	d	a	b	c	d	a	b	c	d
Methylcholanthrene applied to skin four times before inoculation*	1:50,000	±	++	±	++	+++	++++	+++	+++±	+++±	++++	+++	+++±
	1:500,000	±	+	±	+	±±	++	+	++	++	++	+	++
	1:5,000,000	0	±	0	0	+	+	±	±	+	±±	±	±
Methylcholanthrene applied to skin four times after virus inoculation†	1:50,000	±	0	0	±	±	+	0	±	±	+	0	±
	1:500,000	0	0	0	0	0	0	0	0	0	0	0	0
	1:5,000,000	0	0	0	0	0	0	0	0	0	0	0	0
Normal skin	1:50,000	±	0	0	0	++	+	±	0	++	+	±	0
	1:500,000	0	0	0	0	±	0	0	0	±	0	0	0
	1:5,000,000	0	0	0	0	0	0	0	0	0	0	0	0

* 0.3 per cent methylcholanthrene in benzene applied to skin four times at 2 day intervals before virus inoculation.

† 0.3 per cent methylcholanthrene in benzene applied to skin four times at 2 day intervals beginning 5 days after virus inoculation.

1:500,000, and 1:5,000,000. One area of each row received the same dilution of virus. Five days later, when the skin areas had almost healed, one of the rows of patches which had not been treated prior to inoculation was painted with 0.3 per cent methylcholanthrene in benzene, and the applications were kept up at 2 day intervals for a total of four times, again inducing the familiar changes. To the other rows of patches nothing further was done.

The results of the experiment are set down in Table VII. On the 18th day after inoculation the virus diluted 1:500,000 had caused papillomas on all of the patches treated with methylcholanthrene prior to inoculation and so too had a dilution of 1:5,000,000 in one rabbit. The control patches and those treated with methylcholanthrene after inoculation showed at this time no

papillomas where virus had been inoculated in dilutions above 1:50,000, and they were only just appearing where this latter had been put. By the 35th day (after which no growths appeared) the 1:5,000,000 dilution of the virus had produced papillomas on all of the skin areas treated before inoculation, but had failed to cause growths on untreated areas or on those that were treated with methylcholanthrene after virus inoculation. 1:50,000 caused large, confluent or semiconfluent papillomatous masses on all of the areas prepared before inoculation, but it produced only a few discrete growths on the other areas.

Manifestly, methylcholanthrene treatment of the skin after virus inoculation did not enhance the effects of the virus. A similar experiment, using a mixture of turpentine and acetone instead of methylcholanthrene, yielded similar results except that fewer growths arose on the skin treated with the turpentine and acetone after scarification than on the untreated skin.

Results of Altering the Skin of Cottontail Rabbits

The work was now extended to cottontail rabbits, the natural hosts of the papilloma virus, to learn whether their skin can be rendered more susceptible to the virus.⁴

Several experiments of the sort already described were frustrated by skin injury, maceration and bacterial infection occurring when two to four applications of methylcholanthrene or the mixture of turpentine and acetone were applied at 2 day intervals, —procedures well tolerated in the domestic rabbit. However, when the intervals were lengthened to 4 days the skin underwent the same gross changes as in these latter animals. But unfortunately it also became notably susceptible to bacterial infection and scarification frequently resulted in broad abscesses. To avoid these, resort was had to intradermal inoculations of the virus. These gave irregular results, yet by and large the several experiments gave clear indications that methylcholanthrene, benzene, and a mixture of turpentine and acetone, all render the epidermis of the cottontail rabbit unusually susceptible to the papilloma virus.

Nature of the Skin Changes

The agents that enhanced the susceptibility of the skin for the papilloma virus all caused marked alterations of the same general sort. In the gross they consisted of thickening, scurfing, increased pigmentation, and more or less

⁴ The papilloma virus ordinarily titers slightly higher in susceptible cottontails than in the domestic species. Table VIII shows the results of rubbing graded dilutions of a virus extract (W.R. 1-28) into scarified skin areas of six normal domestic rabbits and six cottontail rabbits. One cottontail proved immune to the virus and hence was not included in the table. It will be seen that the virus extract produced growths in all of the cottontail rabbits in dilutions up to 1:800,000, whereas this last caused none in any of the domestic rabbits, and in only two of the six did a dilution of 1:400,000 cause any.

acute inflammation. The extent of the changes, however, varied with the agent used.

The microscopic findings can be briefly summarized:—

The *normal skin* of the abdomen of the domestic rabbit has a thick connective tissue stroma and a mere skim of superficial epidermis (Fig. 1), consisting of a Malpighian layer one to three cells thick which keratinizes abruptly without differentiation. The hair follicles do not go deep and the sebaceous glands are small and inconspicuous. Three to four applications of *tar* to the skin were found to cause striking changes (Fig. 2). The Malpighian layer became greatly thickened and the cells appeared larger than normal and showed many mitoses. They differentiated gradually, forming a granular layer and a thick stratum of keratinization. The hair follicles became greatly distended with keratin in many instances. Numerous sebaceous glands appeared and the dermis showed an acute inflammatory reaction with edema, small hemorrhages, and cellular infiltration. All these changes have been often described before. A single application of *methylcholanthrene* caused a mild inflammatory reaction in the dermis and the basal cells of the epidermis appeared to be slightly larger than normal but were not increased in number. After three or four applications the epidermis was markedly hyperplastic and the changes were similar to those seen in the tarred skin, but lesser in degree (Fig. 8). After six applications there was even greater thickening and irregularity of the epidermis and portions of the skin were necrotic. When the treatments were discontinued the skin gradually reverted to the normal. Within 1 week after the skin had received four applications of methylcholanthrene, regression was evident. The epidermis was still thicker than normal and exhibited a graded differentiation but the cells showed far fewer mitoses than 24 hours after the treatments and were smaller and less abnormal. Within 2 weeks involution was advanced, and after 3 weeks the epidermis had returned to normal save for a slight hyperkeratosis. The hair follicles appeared normal now except at the bases, where there was still some thickening of the epithelial layer and the cells showed numerous mitoses and were slightly larger than normal. A few polymorphonuclear leucocytes could still be seen in the connective tissue. After 4 weeks the skin seemed wholly normal. *Benzpyrene* caused changes similar to those induced by methylcholanthrene except that the sebaceous glands were more hyperplastic and occasionally cystic.

The non-carcinogenic agents which were effective in increasing the susceptibility of the skin for the virus brought about much the same histological changes. *Turpentine* and *acetone* caused less hyperplasia of the epidermis than methylcholanthrene (Fig. 3) but a more acute inflammatory reaction in the subcutaneous tissue. The *benzene-treated skin* was less hyperplastic than that altered by methylcholanthrene, but the general changes were similar. Occasionally there were small patches of epidermal necrosis. The sebaceous glands were markedly hyperplastic and showed differentiation.

Ultraviolet light caused an acute inflammatory reaction 48 hours after irradiation, but the epidermis was still as thin as usual at this time and sometimes showed small areas of necrosis. *X-ray irradiation* elicited no significant changes in the skin under the conditions employed (Experiment 3). These agents failed to alter the susceptibility of the skin for the virus, as Experiments 3 and 4 showed.

The microscopic changes elicited in cottontail rabbit skin by the carcinogenic and non-carcinogenic agents were at least as marked as those seen in the domestic rabbit and usually more so. Two to three applications of methylcholanthrene, benzene, or turpentine and acetone at intervals of 4 days changed the epidermis of the wild rabbit from a thin, delicate epithelium one to two cells thick, to a greatly thickened and irregular, differentiating sheet, with distended, hyperplastic hair follicles and numerous sebaceous glands. Further applications usually rendered the epidermis necrotic.

The findings plainly showed that the various agents which enhanced the susceptibility of rabbit skin all caused the epidermis to become hyperplastic and to proliferate actively. The agents which proved ineffective,—ultraviolet light and x-ray,—did not do so, although the former produced an acute inflammatory reaction in the skin.

The Skin Changes Induced by Scarification

Several authors have described the early stages in virus-induced papillomatosis (9) but no one has inquired into the changes which follow immediately upon scarification of the skin and virus inoculation. It seemed probable that a knowledge of these changes would aid toward an understanding of the reasons for the increased susceptibility of altered skin, and consequently a study of them was undertaken. Papilloma virus was rubbed into some scarified areas and a solution of minute graphite particles (hydrokollag (10)) into others, to learn the fate of particulate matter as such.

Experiment 9.—Nine skin areas on the abdomens of three domestic rabbits were painted with 0.3 per cent methylcholanthrene in benzene at 2 day intervals for a total of four times. 24 hours after the last treatment a representative piece of the changed skin was removed from each animal. All of the areas were then scarified with sandpaper to the usual extent, that is to say, until there was oozing of serum, sometimes blood-tinged, and a 5 per cent papilloma virus extract (W.R. D) was rubbed into three of the patches, a dilute suspension of hydrokollag in saline into three others, while the remaining three areas were left as such. In addition nine untreated areas on each of three other rabbits were similarly scarified and the virus and the hydrokollag suspension were rubbed into them. Slices of skin were taken from all of the various areas 5 hours after scarification, and additional pieces were procured after 1, 2, 4, 6, 8, 11, and 14 days. They were fixed in acid Zenker's and stained with eosin and methylene blue. Duplicate sections from the areas receiving hydrokollag were stained with Lichtgrün as well.

It was found that scarification of the *normal skin* to the extent ordinarily employed for virus inoculation removed practically all of the epithelial covering and some of the superficial connective tissue as well, cutting across the hair follicle shafts near the surface. Only occasional small islands of surface epidermis were left. After 24 hours the connective tissue was edematous and showed a few polymorphonuclear leucocytes and a thin scab had formed on the surface, but as yet no epithelial repair had taken place. The scab had become much thicker after 24 hours, owing not only to the ac-

cumulation of dried exudate but mostly to necrosis of the superficial connective tissue layer with incorporation of the dead material in the scab. After 48 hours epithelial regeneration was for the first time plainly evident. It took origin almost entirely from the cells of the hair follicle shafts, the epithelial cells extending laterally between scab and living connective tissue and sometimes into crevices in the latter. The first extension seemed to be mostly migration though occasional mitoses could be seen. The newly formed cells showed many mitoses later. After 2 to 4 days they had multiplied and spread laterally to such extent as to form umbrella-like expanses with the hair follicles as the staffs of the umbrellas. At this time the epidermis had not nearly covered all of the denuded surface. The connective tissue was still edematous and showed some round cells and polymorphonuclear leucocytes but the scab had begun to separate here and there. After 4 to 8 days the surface was entirely covered with a layer of hyperplastic epithelium (Fig. 5) three to six cells thick which had differentiated into granular and keratinized layers. The picture was markedly different from the normal (Fig. 1). The hair follicle shafts had thickened only slightly except next the orifices where the epithelium was hyperplastic. There was no evident stimulation of the sebaceous glands. At this time the surface scab had only just come away and remnants of it could be seen. Later the hyperplastic epidermis slowly took on a normal appearance and by the 14th day it had nearly done so, though here and there it was still slightly thickened and hyperkeratotic.

The skin areas inoculated with papilloma virus showed similar general changes during the first days, and with the inoculum employed,—which gave rise to growths relatively late,—changes referable to the virus were not discerned until 6 to 8 days had elapsed. Then the characteristic alterations of early papillomatosis (9) could be made out here and there. By the 11th to the 14th day there were discrete characteristic growths. The microscope showed most of them to have arisen from the basal layers of the new hyperplastic epidermis which now covered the surface between the hair follicles.

The areas into which hydrokollag had been rubbed showed the black particles in close contact with the abraded surface immediately after the inunction and in direct contact with the epithelium of the hair follicles that had been cut across. But as the scab thickened, owing to the necrosis of the superficial tissue with incorporation in it of the dead layer, almost all of the hydrokollag became incorporated too, only traces remaining in crevices here and there where it might come in contact later with the regenerating epithelial cells. Careful search was made for phagocytosis of it by these elements but none could be found, and when the newly formed epidermis keratinized, by the 4th to the 8th day after scarification, it was all cast off.

These findings show that the scarification of normal skin as ordinarily done removes nearly all of the surface epidermal cells and entails a loss of most of the virus (if one can judge from what happens to hydrokollag) in the scab which forms, the result being that the chance for it to reach susceptible cells is greatly reduced. Almost the only spots at which virus is directly brought into contact with epithelium are where the hair follicles have been cut across and even here the epithelium commonly becomes implicated in the later

necrosis and is lost. Papillomas derived from the hair follicle epithelium are relatively infrequent. Instead they usually appear on the surface between the hair follicles and one must suppose they principally arise from young, regenerating cells which have extended laterally from the follicles to cover the denuded surface.

Scarification of the *methylcholanthrene-treated skin* (Fig. 8) was done to the same extent as with normal skin, that is to say, until the surface of the patch seemed everywhere abraded and serum oozed out. Though the surface epithelium was markedly hyperplastic, the microscope showed that practically all of it had been removed as in the case of the normal skin, exposing the fibrous corium and the hair follicle shafts (Fig. 9). These latter were hyperplastic. The connective tissue became edematous as usual, polymorphonuclear leucocytes wandered into it, and scabbing took place with increase in the thickness of the scab by superficial necrosis (Fig. 10). But epithelial regeneration was far more rapid than under ordinary circumstances. Within 24 hours after scarification it was already far advanced (Fig. 10) and within 48 hours the entire abraded surface was covered (Fig. 11). Migration began by lateral extension from the hair follicles, and was attended by such active multiplication that very soon large, thick, umbrella-like structures with the follicles as the shafts of the umbrellas had formed beneath the surface scab (Fig. 12). They consisted of great numbers of epidermal cells, many of them in mitotic division, and as these spread along the surface beneath the scab they frequently invaded irregularly the crevices in the fibrous corium. The sheet of hyperplastic, actively regenerating epidermis that rapidly formed was much thicker than that produced by the regeneration of ordinary epidermis (Fig. 5) and consisted of a shallow keratinized layer and a Malpighian layer ten to fifteen cells deep, showing numerous mitoses. Irregular papillae extended down into the connective tissue. The hair follicles were distended with keratinized epithelium and the epithelial lining was thickened. There appeared to be a notable increase in the number of sebaceous glands and they were rendered prominent not only by hyperplasia but by retention of secretion which caused many of them to be actually cystic. It seems probable that the hyperplastic surface epithelium had become so crowded through active proliferation as to prevent escape of their contents. Four to 6 days after scarification the epidermis was more orderly, differentiating into wide granular and keratinized layers, and as keratinization progressed the scab came away. Involution took place rapidly and by the 14th day the epithelium had returned practically to normal save for irregularities of basal contour and local thickenings where the layer of living cells was still three to four cells deep. The hyperkeratosis was now in general less than that where the normal skin had been scarified 14 days previously. While involution was going on the sebaceous glands at first became still more cystic, some of them rounding out into small spheres but later the distention of them disappeared and they again became inconspicuous.

The methylcholanthrene-treated areas which were inoculated with virus after scarification underwent similar changes. The effects of the virus were evident in them earlier than when scarified normal skin had received the same inoculum, the characteristic cytological changes indicative of the beginning of papillomatosis being perceptible in one rabbit 4 days after inoculation and on the 6th day in others. The fate of the

hydrokollag was the same as when rubbed into scarified normal skin. Practically all of it was caught in the scab and came away when this did.

From these findings it is plain that the changes which render skin especially susceptible to the virus provide to the latter young, actively proliferating epidermal cells in unusually great number and at a much earlier stage in events than when normal skin has been scarified. Where the hair follicles have been cut across by the sandpaper many more cells are exposed to direct infection with the virus, and where it persists under the scab the regenerating epithelium soon reaches it.

Intradermal Inoculation of Virus into Prepared Rabbit Skin

Despite the defects of inoculation into scarified areas as just disclosed, it has proved the most certain method to the present for titrating the papilloma virus. Intradermal injection of active virus into normal skin does not always result in growths, and the incubation period is regularly longer than in scarified skin (4).⁵ It has seemed worth while nevertheless to determine the effects of the intradermal inoculation of virus in skin altered by methylcholanthrene. Accordingly papilloma virus was so inoculated and was also rubbed into scarified normal and methylcholanthrene-treated skin areas at comparable situations in the same animals. Incidentally, to enlarge the general findings, a mixture of methylcholanthrene and Scharlach R was applied to some areas, and the dye was injected intradermally into other areas that had also been treated with methylcholanthrene prior to virus inoculation. Scharlach R is known to cause a profuse epidermal proliferation and sometimes temporary downgrowths simulating early carcinomatosis (11).

Experiment 10.—Four rectangular areas were clipped on each side of the abdomens of four rabbits. One on each side was painted with 0.3 per cent methylcholanthrene in benzene, another with a saturated solution of Scharlach R in benzene containing 0.3 per cent methylcholanthrene, while a third was painted with 0.3 per cent methylcholanthrene in benzene and 0.1 to 0.2 cc. of a saturated solution of Scharlach R in olive oil was injected intradermally at several places. The treatments were repeated four times at 2 day intervals. The fourth area on each side served as control. Two days after the last treatments pieces were taken from each area of two of the rabbits for microscopic study. The mixture of methylcholanthrene and Scharlach R caused much greater skin alterations than did methylcholanthrene alone. The thickened skin was rendered far more rugose and became so redundant as to be thrown into folds. Microscopically, the changes resembled in many ways those seen in the tarred epidermis, already described, but the thickening of the surface layer of epithelium

⁵ This is also true of tattoo inoculations.

TABLE VIII

Comparison of the Infectivity of the Virus in Domestic and in Cottontail Rabbits

Rabbit species	Dilution of virus extract W.R. 1-28	Pathogenicity tests																	
		16th day					28th day						42nd day						
		a	b	c	d	e	f	a	b	c	d	e	f	a	b	c	d	e	f
Test rabbits.....																			
Domestic rabbits	1:10,000	0	0	±	±	+	++	++	±	±	++	++	++	±	±	±	++	±	+++
	1:100,000	0	0	0	±	0	±	0	+	±	++	+	+	±	+	++	±	±	++
	1:400,000	0	0	0	0	0	0	0	0	±	0	+	+	0	0	0	±	0	+
	1:800,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Test rabbits.....		g	h	i	j	k		g	h	i	j	k		g	h	i	j	k	
Cottontail rabbits	1:10,000	±	±	+	++	±		++	++	++	++	++		++	++	++	++	++	
	1:100,000	±	±	0	+	0		+	+	+	++	±		+	±	+	++	±	
	1:400,000	0	0	0	±	0		±	0	+	+	±		±	±	+	+	±	
	1:800,000	0	0	0	0	0		0	0	±	±	±		±	±	±	±	±	

TABLE IX

Comparison of Intradermal Virus Inoculation and Virus Inunction after Scarification of Normal and Prepared Rabbit Skin

Method of inoculation of virus extract W.R. 1-28, 1:500,000	Skin treatment before virus inoculation	Pathogenicity tests											
		20th day				28th day				35th day			
		a	b	c	d	a	b	c	d	a	b	c	d
Intradermal	Normal (untreated)	0	0	0	0	0	0	0	0	0	0	0	0
	Methylcholanthrene*	±	+	±	±	+	+	+	±	+	++	+	±
	Methylcholanthrene and Scharlach R†	+	+	+	+	++	+	++	+	++	++	+	+
	Methylcholanthrene and intradermal injections of Scharlach R‡	+	±	±	+	++	+	±	++	++	++	++	++
Scarification	Normal (untreated)	0	0	0	0	0	0	±	±	0	0	±	±
	Methylcholanthrene*	++	±	±	0	++	+	++	±	++	+	++	+
	Methylcholanthrene and Scharlach R†	±	±	±	±	±	+	++	++	±	+	++	+
	Methylcholanthrene and intradermal injections of Scharlach R‡	±	++	±	±	+	++	++	+	+	++	++	++

* 0.3 per cent methylcholanthrene in benzene.

† Saturated solution of Scharlach R in benzene containing 0.3 per cent methylcholanthrene.

‡ 0.3 per cent methylcholanthrene in benzene plus intradermal injections of a saturated solution of Scharlach R in olive oil.

was not so considerable (Figs. 2 and 4). The intradermal inoculation of Scharlach R in olive oil into the the methylcholanthrene-treated skin caused small nodules at the points of injection, consisting of much new formed connective tissue about the oil globules and what appeared to be malignant downgrowth in one instance,—spurious carcinomatosis as the event proved.

A virus extract (W.R. 1-28) diluted 1:500,000 in saline was inoculated into the patches 24 hours after the last treatment. It was rubbed into the normal and treated skin areas on one side of the abdomen after the usual scarification and injected intradermally (0.1 to 0.2 cc. in eight places) into those of the other side.

Table IX shows the results of the experiment. No papillomas resulted from the intradermal inoculation of the very dilute virus into normal skin, but where it had been rubbed into scarified areas a few growths slowly appeared in two of the four rabbits. The areas treated with methylcholanthrene or methylcholanthrene and Scharlach R, on the other hand, all yielded discrete papillomas in more or less considerable number and in general they were as numerous and grew as fast on the intradermally injected areas as on those that were scarified.

In this experiment the addition of Scharlach R, though producing more marked skin changes, failed to increase the susceptibility of the methylcholanthrene-treated skin to virus rubbed into it after scarification. It seems possible that methylcholanthrene brings about maximal susceptibility to the virus. When the virus was introduced intradermally into hyperplastic epidermis, it produced as many growths as when rubbed into the hyperplastic skin following scarification.

DISCUSSION

The experiments make plain that those methods of papilloma virus inoculation which result in growths all provide young, actively regenerating cells in abundance. Cells in this condition would appear to be essential for infection. As already stated, normal skin can be infiltrated with large amounts of highly pathogenic virus and yet no papillomas be caused. Furthermore when virus-containing fluid is injected in quantity into normal skin, producing a broad wheal, papillomatosis arises only where the needle has caused injury although the epidermis over a considerable area must have been brought into contact with the inoculum. The experiments of Kidd and Rous (7) have already been mentioned, in which normal rabbit ears were infiltrated with virus fluid by way of a marginal vein, without result in growths save at points of injury. Tarring the ears within the next 7 days, however, to such extent as to induce hyperplasia, brought out a horde of growths. One may recall in this general connection that bacteriophage multiplies only in the presence of young, actively growing bacteria (12) and that the first evidence of infection with vaccine

virus, herpes virus, and virus III is found in the young cells filling in the defects consequent on scarification (13).

The experiments here reported to learn the effects of the scarification preliminary to virus inoculation and the stages in its repair have disclosed that most of the surface epithelium is scraped away and that practically all of what is left, and the superficial connective tissue as well, is destroyed in the scab which forms within the next 2 days. As already pointed out, the virus rubbed into the raw surface must also be lost in the scabbing and but little can remain here or there underneath the dead material to infect the young, regenerating epidermal cells which extend from the hair follicles. It is possible that some direct infection of the preexisting hair follicle cells may occur at the time when the shafts of the follicles are cut across by the sandpaper and the virus is rubbed in, but this seems unlikely because the scabbing involves them too. Indeed, microscopically one seldom sees papillomas beginning at the hair follicles, these ordinarily arising from the reconstituted surface sheet of epithelium which lies between them.

The various agents which render the skin more susceptible to infection with the virus all effect changes which should bring young, actively regenerating cells into association with the virus in much greater number than ordinary and at a much earlier time. They also increase the local vascularity, and to this circumstance as well as to a much more abundant initial cell infection the shortened incubation period and subsequent rapid enlargement of the papillomas can be laid. Olitsky and Schlesinger (14) have recently shown that local edema produced by the subcutaneous injection of hypertonic solutions prior to inoculation of the skin with herpes virus greatly increases the effectiveness of the latter; and Sprunt (15) has brought evidence to show that susceptibility to infection with vaccine virus is influenced by the number of cells exposed to the latter. But the increased susceptibility of the altered skin for papilloma virus may be due to more than the provision of a richer vascularization and of an increased number of susceptible cells for infection. The individual young cells of an epidermis regenerating after alteration by the preparatives of the present work may be especially susceptible to the virus.

Under the ordinary circumstances of scarification and virus inoculation infection takes place at scattered points with secondary coalescence, the result being separate more or less broad-based papillomas or confluent papillomatous expanses. The scattered character of the initial infection explains why confluent masses usually have craggy peaks with clefts between that frequently extend down to the skin level, and why on cross-section local differences can be perceived, which are expressions of the proliferation of differing infected cell families (16). Only occasionally does infection by scarification result in growths so compacted as to suggest that a broadcast, primary infection has

taken place. Naturally occurring papillomas in cottontails are not infrequently compacted, however, having the form of solid discs or "onions," presumably because they are the outcome of primary punctate infection with expansile enlargement. Growths of similar sort can be produced experimentally in both cottontails and domestic rabbits by intradermal or tattoo inoculation.

Bryan and Beard (17) have lately laid stress on the length of the incubation period (time elapsing after inoculation before papillomas are visible in the gross) as a reliable indicator of the amount of active virus present in the inoculum. There can, of course, be no doubt that the greater the number of virus entities distributed upon a scarified area the greater the chance will be for cells to become infected, up to a certain maximum, other things being equal and,—since most papillomas are consequent on multiple cell infection,—the sooner should the papillomas appear. But if the skin is abnormal other factors enter into the matter, as the present work shows, notably the regenerative activities of the epidermis and the local vascular state. The rate of appearance of papillomas is conditioned not only by virus quantity and pathogenicity but by the state of the tissue acted upon.

The experiments here reported provide a practicable method to render papilloma virus many times more effective on experimental inoculation. Papilloma extracts that do not elicit growths in normal skin in dilutions beyond 1:100,000 produce growths in methylcholanthrene- or turpentine and acetone-treated skin in dilutions of 1:1,000,000 or 1:10,000,000, while furthermore the incubation period of the papillomas in the altered skin is considerably shorter than in normal skin. It has been calculated that about 94,000,000 papilloma protein molecules are present in the dilution giving the 50 per cent point (18). The findings of the present experiments suggest that this figure mostly expresses the difficulty of bringing virus into association with susceptible cells. By the means described in the present work the number of entities in the inoculum requisite to infection can be reduced considerably, although under the circumstances of virus inunction into scarified skin most of the virus is still lost by the way. To gauge the effectiveness of individual papilloma virus entities one would have to be certain that these reached the appropriate cells and that the latter were in a state to be infected. Needless to say, these considerations apply to other viruses as well.

Experiments carried out with Dr. John G. Kidd have shown that skin preparation by the methods here described is useful in demonstrating papilloma virus in materials which fail to give rise to growths when inoculated in the ordinary way. Extracts of domestic rabbit papillomas which are non-pathogenic when so tested, in which, that is to say, the virus appears to be "masked," produce growths in most instances when inoculated into methylcholanthrene-

or turpentine and acetone-treated skin. These findings will be reported in detail in a forthcoming paper.

SUMMARY

Rabbit skin can be rendered abnormally susceptible to papilloma virus infection by preliminary treatments with a variety of agents. The most effective agents thus far found are 0.3 per cent methylcholanthrene in benzene and a mixture in equal parts of turpentine and acetone, applied four or five times at 2 day intervals. When virus is inoculated into skin altered by these agents, either intradermally or by inunction after scarification, papillomas appear earlier and in greater number than on normal skin, and much higher dilutions give rise to growths. The method provides a means of detecting amounts of virus which cause no papillomas upon inoculation into normal skin.

Papilloma virus material which is rubbed into scarified normal or hyperplastic skin is largely lost in the scabbing which ensues, and nearly all of it fails to reach susceptible cells. The preparatory agents which increase the effectiveness of the virus bring about marked epidermal hyperplasia, and the hyperplastic tissue regenerates with greater rapidity when scarified. The agents evidently act in large part by providing young epidermal cells in quantity to the virus, as also by inducing a richer vascularization than ordinary in support of the papillomatous proliferation. It is possible that they also act by providing especially susceptible cells. The implications of the findings are discussed.

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EXPLANATION OF PLATES

All of the specimens were stained with eosin and methylene blue.

The photographs were made by Mr. Joseph B. Haulenbeek.

PLATE 2

FIG. 1. Normal skin, from the abdomen of a normal domestic rabbit. It has been overstained with hematoxylin to make the thin epidermal layer more evident. $\times 18$.

FIG. 2. Portion of an area of abdominal skin which had been tarred twice a week for 2 weeks. Biopsy 2 days after last application. $\times 18$.

FIG. 3. Skin treated with turpentine and acetone. The mixture had been applied five times at 2 day intervals. Biopsy 24 hours after last treatment. $\times 18$.

FIG. 4. To show the skin alterations induced by four applications at 2 day intervals of a saturated solution of Scharlach R in benzene containing 0.3 per cent methylcholanthrene. $\times 18$.

FIG. 5. Illustrating the marked hyperplasia of normal skin after scarification. Specimen taken 8 days after scarification. $\times 18$.

FIGS. 6 and 7. Cross-section of the papillomatous masses formed after broadcast tattoo inoculation of normal skin (Fig. 6) and of methylcholanthrene-treated skin (Fig. 7) of the same rabbit. The growths were removed and sectioned 42 days after virus inoculation. Only a portion of the mass on the methylcholanthrene-treated skin is shown. $\times 2\frac{1}{2}$.



(Friedewald: Cell state as affecting susceptibility to virus)

PLATE 3

FIGS. 8 to 12. To illustrate the changes induced by scarification of methylcholanthrene-treated skin. All the specimens were taken from the same rabbit.

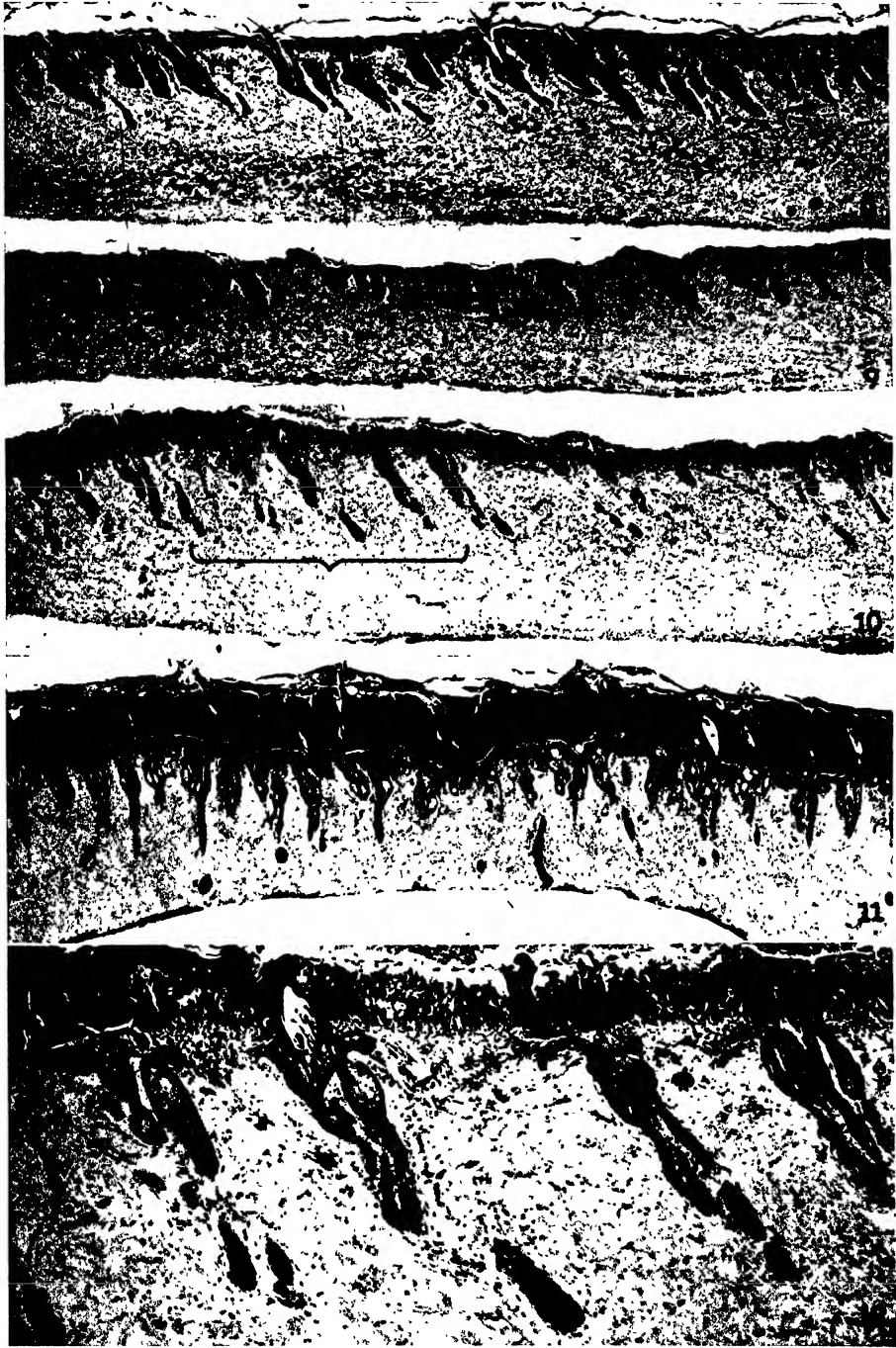
FIG. 8. Methylcholanthrene-treated skin before scarification. 0.3 per cent methylcholanthrene in benzene had been applied to it four times at 2 day intervals. Biopsy 24 hours after last treatment. $\times 18$.

FIG. 9. 5 hours after scarification. The surface epithelium has been almost completely removed together with some connective tissue and the upper portion of the hair follicle shafts. A scab is forming on the surface. $\times 18$.

FIG. 10. 24 hours after scarification. The scab is unusually thick as result of superficial necrosis involving both the connective tissue and hair follicle shafts. From the latter epithelial regeneration is already taking place, the new cells extending between scab and connective tissue. The bracketed region is shown at greater magnification in Fig. 12. $\times 18$.

FIG. 11. 48 hours after scarification. Epithelial regeneration is now complete and the denuded surface is covered with markedly hyperplastic epidermis. The sebaceous glands are also hyperplastic and many are distended with secretion. Hence their prominence as compared with those of Fig. 8. $\times 18$.

FIG. 12. The bracketed region of Fig. 10 at higher magnification. The epithelium is spreading laterally from the shafts of the hair follicles to form umbrella-like structures. Some of the new epidermal cells can be seen extending irregularly into crevices in the fibrous corium. $\times 60$.



(Friedewald: Cell state as affecting susceptibility to virus)

SEROLOGICAL REACTIVITY OF HYDROLYTIC PRODUCTS FROM SILK

By K. LANDSTEINER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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It has been briefly reported that precipitin reactions of antisera produced with heteroprotease are inhibited by peptic proteoses, small enough in molecular size to pass collodion membranes (1, 2). The purpose of these investigations was to get information on the smallest portions of a protein molecule that still are capable of specifically combining with antibodies and, finally, on the chemical structure of these determinant groups, questions as yet undecided. In resuming this line of work split products of silk, chosen as an example of a fibrous protein with a comparatively simple amino acid make-up, were examined and again inhibition reactions were observed with substances resulting from enzymatic digestion of this protein (see Bergmann and Niemann, 3). It was further seen that such reactions are obtained also with products of acid hydrolysis.¹

The preparation of antisera for silk presented some difficulty at first but on injection of silk, dissolved with the aid of acid and after neutralization adsorbed onto charcoal, immune sera were obtained (4a). An attempt with the method briefly mentioned by Fell (4b) was unsuccessful.

Methods and Materials

Immunization.—6 gm. of degummed silk were dissolved by stirring for about 4 minutes in 60 cc. concentrated hydrochloric acid, and the solution diluted with 10 volumes of water. The solution was neutralized to weak acid reaction to Congo red with 10 per cent NaOH, added in portions to avoid too high a temperature. The solid material which separates was allowed to settle and the supernatant fluid syphoned off. The sediment was washed by suspending in a large volume of water and after settling was centrifuged for a short time—at very low speed to prevent the formation of clumps that subsequently would be difficult to bring into solution. The precipitate was then taken up in 45 cc. of water and dissolved by adding dropwise dilute NaOH while stirring, avoiding an excess. The solution was brought back with dilute HCl to weakly alkaline litmus reaction.² This solution was used for the sero-

¹ This observation was made in our laboratory by Dr. R. F. Clutton.

² An electrophoretic experiment was kindly carried out by Dr. L. G. Longsworth. The opalescence of the solution interfered with the observation but the material seemed to be homogeneous and had a mobility of -1.8×10^{-5} cm./sec./volt/cm. in 1 per cent solution in phosphate buffer of 0.1 ionic strength and pH 8.0.

logical tests and for immunization after adsorption to blood charcoal. The charcoal was washed several times with water, dried, sterilized, suspended in saline solution, and thoroughly mixed with the silk solution, using 45 gm. of charcoal for the solution made from 12 gm. of silk (for preservation 0.5 per cent phenol was added). Immunization was carried out by repeated intraperitoneal injection of rabbits, each receiving at 5 day intervals 15 cc. of a 6-fold dilution of the suspension in saline, a dose containing about 25 mg. of silk and 0.35 gm. charcoal. Sera of moderate strength were obtained after 6 to 12 injections.

*Hydrolysis and Fractionation.*³—200 gm. portions of degummed silk were dissolved, with stirring, in 1 liter of 50 per cent (by volume) sulfuric acid, and the solution was allowed to stand at 26–30°C. for 4 hours. 2 liters of water were added, the solution (for convenience) kept overnight in the ice box and then dialyzed in cellophane tubing against running water for 3 hours. The remaining sulfuric acid was then removed with barium carbonate, in later runs with barium hydroxide, and any excess of barium was subsequently removed with sulfuric acid. The barium sulfate was filtered off on Buchner funnels and washed with boiling water. The combined filtrates and washings were evaporated in small portions on the steam bath to a small volume, the reaction during evaporation being kept slightly acid to litmus. The syrupy solution was placed in cellophane tubes and dialyzed in the ice box against 2 volumes distilled water with changes of water at the intervals stated. The outer fluids were evaporated in small portions on the steam bath, and finally were precipitated with acetone, filtered, and dried. The first three diffusates, after dialysis for 3, then 5, then 7 days, were combined. Yield 1200 gm. from 4800 gm. silk used. (The materials obtained on further dialysis are not considered in the following; also, in the description of the fractionation procedures only those substances are taken account of that were selected for use in the serological tests presented.)

The substance was boiled up with 2400 cc. of water, cooled to room temperature, and a little toluene added as a preservative. After standing overnight at room temperature an insoluble portion was removed from the solution (A). This sediment was again extracted with water as above (solution B). To solution A 1½ volumes of alcohol were added and the mixture kept in the ice box for 48 hours. The resulting precipitate was filtered in the cold, washed with acetone, and dried.⁴ From the filtrate a 2nd fraction was obtained by addition of 1/5 volume of alcohol (A₁, 102 gm.). The supernatant was evaporated to a small volume and precipitated with acetone (A₂, 350 gm.). Solution B was precipitated with 2 volumes of alcohol (precipitate B₁, 35 gm.). A second precipitate was obtained by adding 3 volumes of alcohol and the supernatant fluid was evaporated to a small volume and precipitated with acetone (B₂, 100 gm.). A₁ was further fractionated by successive addition of 1, ½, and

³ In studies on the production of silk hydrolysis several dipeptides and a tripeptide (alanylglycyltyrosine) have been isolated in pure state (*cf.* 3). A number of other split products have been described (5, 6).

⁴ The same procedures: dissolving in 2 volumes of water except when stated otherwise, precipitation, drying, were followed in the other fractionations. The volumes of alcohol used in the fractionation are given in terms of the volume of the original aqueous solutions.

1 volume of alcohol, and the last precipitate (11 gm.) was similarly refractionated with 1_2 , 1_2 , and 3_4 volume of alcohol. The 3rd fraction is designated as preparation I.

A₂ and B₂ were joined, dissolved, and the solution precipitated with 3 volumes alcohol; the precipitate (86 gm.) was dissolved in 3 volumes of water and again precipitated with $1\frac{1}{2}$ volumes of alcohol. This substance (35 gm.) dissolved in 4 volumes of water, was separated into fractions which precipitated with $\frac{3}{4}$, 1, 2, and 3 volumes alcohol. The last of these fractions, preparation II, amounted to 1.5 gm.

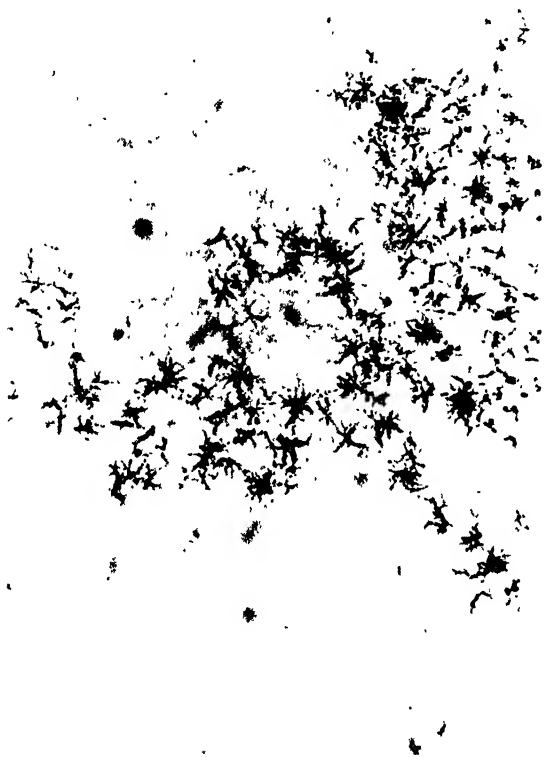


FIG. 1. Substance II from hydrolysis products of silk fibroin. $\times 240$.

The precipitate B₁ was redissolved in 6 volumes of water and precipitated with an equal volume of alcohol (preparation III, 7 gm.).

A number of serologically active preparations were analyzed for total nitrogen and amino nitrogen in order to obtain estimates of the molecular size. The nitrogen analyses were made by the gasometric Kjeldahl method according to Van Slyke (with a modification devised by Goodner⁵). The amino nitrogen determinations were made by the Sørensen method using glass electrode measurements instead of indicators.

⁵ Personal communication.

Glycine was determined with nitranilic acid (7), tyrosine by Bernhart's technique (8), and serine and alanine by the method of Fromageot and Heitz as modified by Desnuelle (9). (Deamination followed by oxidation and colorimetric determination of acetaldehyde.)

Substances Tested.—Substance I had microscopically a crystalline appearance (similar to that shown in Fig. 1) when separating in the cold from solution in dilute alcohol. It contained 17.34 per cent N, 2.47 per cent $\text{NH}_2\text{-N}$, which would correspond to peptides with seven amino acids and with molecular weights of about 600. The ash was 1.9 per cent, containing a considerable proportion of Ca and SO_4 . To obtain an indication of the degree of homogeneity, the material was dialyzed in a cellophane bag against small volumes of water, the outer fluid being removed, and replaced after 18, 47, 90, and 138 hours. The ratio of total N to amino N was 9 in the final inner fluid which contained only 4 per cent of the starting substance and varied from 7.3 in the first to 8.1 in the last diffusate. That the molecular weight values of the fractions (about 580 to 640) calculated from the amino N are of the correct order of magnitude was confirmed by determination of the freezing point depression (made with the second diffusate) which gave even a lower value, but since complete analytical data are lacking an accurate correction for salt (ash 2.9 per cent) was not possible.

Substance II had likewise a crystalline appearance, as shown in Fig. 1. However, an x-ray picture failed to give definite evidence for crystalline structure.⁶ On analysis 17.76 per cent N, 2.26 per cent amino N, (and 2.57 per cent ash) were found indicating octapeptides. Analyses for amino acids gave 48.8 per cent glycine, 35.6 per cent alanine, and 1.2 to 1.3 per cent tyrosine, the latter probably ascribable to some peptide present in small quantity.

Substance III separated from the solution in the form of shiny balls as seen microscopically. Analysis showed 18.11 per cent N, 1.53 per cent amino N, and 0.79 per cent ash, pointing to peptides with a chain length of 12 amino acids.

FINDINGS

The preparations just described were tested serologically by inhibition reactions (Table I).

It is seen that all three substances inhibited the precipitation of silk antigen by the antisera though to somewhat different degrees. (Still other fractions gave positive reactions likewise but were not further studied.) The strongest inhibition was obtained with substance III which from the analytical results appeared to have the largest molecular size. The specificity of the reaction was shown by tests, given in Table II, with several heterologous antiprotein sera and their corresponding antigens in which also a higher concentration of the substances was used. In order to determine whether the activity of

⁶ Strong reflections were observed corresponding to 4.67 and 4.15 Å. u. and a weak reflection at about 3.7, but they may be due to the ash content. For this examination I am indebted to Dr. I. Fankuchen.

TABLE I

To 0.2 cc. of a 1:10,000 silk solution were added 0.05 cc. of solutions of the substances tested for inhibition and then 2 drops of immune serum.

Readings were taken after 15 minutes at room temperature (1st line) and after 1 hour (2nd line).

Solution tested for inhibition	Substance I	Substance II	Substance III	Control
<i>per cent</i>				
1	0 0	0 0	0 0	± +
1/2	f.tr. tr.	f.tr. tr.	0 f.tr.	
1/4	tr. ±	tr. ±	f.tr. f.tr.	
1/8	(±) +	(±) +	tr. ±	

The intensity of the reactions is indicated as follows: 0, f.tr. (faint trace), tr. (trace), tr. (strong trace), ±, ±, +, +±.

TABLE II

Test as in Table I, using 1 drop of the suitably diluted immune sera, and the respective homologous antigens in a concentration of 1/10,000.

Readings were taken after 1 hour at room temperature.

Immune sera against	Substance II		Substance III		Control
	1 per cent	2 per cent	1 per cent	2 per cent	
Chicken ovalbumin No. I diluted 1:3	±	±	±	±	±
No. II diluted 1:2	+±	+±	+±	+±	+±
Horse globulin No. I diluted 1:3	±	±	±	±	+
No. II undiluted	+	+	+	+	+

preparation III might be due to a substance of high molecular weight, a diffusion experiment was carried out in the following manner.

6 gm. of the substance were dissolved in 10 volumes of water by heating, cooled to room temperature, and some undissolved material (0.315 gm.) filtered off. The solution was placed in a cellophane bag and dialyzed in the ice box against $1\frac{1}{2}$ volumes of water which was changed at the intervals stated. The diffusates were evaporated to a small volume and precipitated with acetone.

	Taken after	Weight	Total N	Amino N	$\frac{N}{\text{Amino N}}$
	<i>hrs.</i>	<i>gm.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1st diffusate	18	2.2	17.54	1.67	10.5
2nd "	24	1.44	17.78	1.56	11.4
3rd "	30	0.82	17.60	1.47	12.0
4th "	43	0.45	17.51	1.51	11.6
5th "	74	0.27	17.19	1.30	13.2
Residue in bag		0.25	16.76	1.08	15.5

Fractions 2 and 3 were analyzed for individual amino acid and gave the values shown. (R = ratio of nitrogen of each amino acid to NH_2 nitrogen in the original substance, indicating the number of molecules of the amino acid.)

	2nd diffusate		3rd diffusate	
	<i>per cent</i>	<i>R</i>	<i>per cent</i>	<i>R</i>
Glycine.	49.7	6.0	46.8	5.9
Alanine	26.4	2.7	25.3	2.7
Serine*	21.2	1.8	22.4	2.0
Tyrosine	18.6	0.9	18.1	1.0

* This value would include other hydroxyamino acids (threonine) (*v.* 10).

Although the substance III is certainly not homogeneous, it would appear that the fractions 2 and 3, which are similar in composition, consist largely of peptides with a chain length of twelve amino acids, and this receives support from the amino acid composition which, incidentally, is not far from the proportions found in silk fibroin⁷ (Bergmann and coworkers, (11, 12)).

The results of inhibition tests with the fractions obtained by diffusion are given in Table III and show that there was no definite difference in the reactions of the five diffusates; the residue gave a somewhat stronger inhibition. On the whole the experiment renders it very improbable that a small quantity of a substance with high molecular weight was responsible for the specific inhibitions observed. Thus the analytical result showed that some separation with respect to molecular size had been obtained, as indicated by the figures for

⁷ A non-crystalline substance, probably a tetrapeptide isolated by Fischer and Abderhalden (5) contained two glycine, one alanine, and one tyrosine residues.

the 1st and 5th diffusates, while this difference is not reflected in the inhibition effects.

TABLE III

Inhibition tests with diffusates of substance III.

Tests as in Table I.

Diffusates	1	2	3	4	5	6	Control
<i>per cent</i>							
1/2	0 f.tr.	0 f.tr.	0 f.tr.	0 f.tr.	0 f.tr.	0 0	+
1/4	<u>tr.</u> <u>tr.</u>	<u>tr.</u> <u>tr.</u>	tr. tr.	tr. tr.	tr. tr.	f.tr. f.tr.	
1/8	± +	± +	± +	<u>tr.</u> +	± +	<u>tr.</u> +	

Continuation of the work in the direction of obtaining more homogeneous preparations of silk peptides is desirable as well as further investigations on other proteins.

The author is indebted to Mr. B. Meier for his assistance.

SUMMARY AND CONCLUSIONS

The foregoing experiments show that products of the hydrolysis of silk that consist of peptides having, from amino nitrogen determinations, molecular weights from about 600 to 1000 were capable of inhibiting the reactions of precipitin sera for silk. From the results it may reasonably be inferred that silk fibroin contains determinant structures not larger than the peptides examined, which probably consist of not more than 8 to 12 amino acids. That similar relations may obtain with other proteins is not improbable in view of results previously reported with dialyzable split products.

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THE ASSOCIATION OF BLOOD CELL FACTORS WITH THE TRANSPLANTABILITY OF THE BROWN-PEARCE TUMOR*

By ALBERT E. CASEY, M.D., LOUISE PEARCE, M.D., AND PAUL D. ROSAHN, M.D.†

(From the Laboratories of The Rockefeller Institute for Medical Research, New York, the School of Medicine of St. Louis University, St. Louis, Mo., and the School of Medicine of Louisiana State University, New Orleans, La.)

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Previous studies have shown wide variations (1), chiefly related to breed (3), in the reaction of individual rabbits to inoculation with the Brown-Pearce tumor. In some breeds less than 10 per cent of the animals were susceptible, but in others more than 90 per cent became riddled with metastases and died within a very short time after transplantation of the tumor.

It has long been suspected that various blood and tissue cells are concerned in the cellular reactions occurring about neoplastic cells. Alterations in the levels of the hemoglobin and of the various blood cells, for instance, have been observed to correspond with the progress or retrogression of neoplasia and with the cellular reaction about the growths. It has been difficult, however, to obtain proof which would demonstrate whether the cellular response in the blood and the tissue about the tumor foci is the cause of the progress or regression of the artificially transplanted tumor or the metastatic tumor, or is itself the result of the progress or regression (7, 8, 11-20, 22-25, 28).

A new approach is clearly necessary to determine the nature of this relationship, and the present experiments have been designed with this objective. They are based upon definitive studies of normal pretransplantation blood levels in individual animals which have been analyzed in relation to post-transplantation reactions. To state the premise differently, it was thought possible that the transplantability, growth, and spread of the Brown-Pearce tumor might be influenced by factors reflected in the pretransplantation blood formulae. The approach would seem to be entirely reasonable, since pre-transplantation levels obviously cannot be influenced by the tumor or its products. It is only recently that the characteristics and hereditary nature of the blood formulae in individual animals have been recognized (2, 5), and previous investigations had therefore not included studies of pretransplantation results, without regard to possible alterations in the blood levels after transplantation of the tumor.

This paper is concerned with the relation of nine blood cell factors (red blood

* This investigation was aided by a grant from The International Cancer Research Foundation.

† Now at Yale University School of Medicine, New Haven, Conn.

cells, hemoglobin, platelets, total white blood cells, neutrophils, basophiles, eosinophiles, lymphocytes, and monocytes) to the success of transplantation of the Brown-Pearce tumor.

Material and Methods

The study was begun with 195 young adult male rabbits and was concluded with 108 animals selected on the basis of rigid criteria to be described later. Seventeen sets of experiments were carried out on these animals between the years 1927 and 1937.

The animals originally selected were mature and apparently healthy, as evidenced by body weight and the size of the testes, as well as by the absence of canker, snuffles, mange, and diarrhea. They ranged in age from 5 to 12 months. Sixty-one were from standard breeds, as follows: Havana 14, Himalayan 12, English 9, Dutch 5, Belgian and Chinchilla 4 each, French Silver 3, Flemish Giant, New Zealand Red, and American Blue 2 each, and Sable, Castor Rex, Polish, and Lilac 1 each. Of the 47 hybrid animals, 39 were common brown-grays purchased from dealers (possibly mixtures of New Zealand, Belgian, Flemish, and Chinchilla) and 9 were hybrids reared in the laboratory from the standard breeds listed.

The food of the 35 brown-grays used in the first 8 experiments was limited to cabbage, lettuce, hay, and oats, and the water intake was restricted. The food of the 73 animals used in the remaining 9 experiments consisted of standard rabbit chow containing various vitamins and essential food elements, and free access to food and water was permitted at all times.

Prior to inoculation with the Brown-Pearce tumor a series of blood studies was carried out as follows:

In 2 sets of experiments involving 14 animals the entire examination was carried out the same day. The examination consisted of 4 red blood cell determinations, 4 hemoglobin and platelet determinations, 5 white blood cell determinations, and 5 differential counts in which a total of 1,000 cells was counted.

In the remaining animals the examinations, which varied from 2 to 12 per animal, were conducted over periods varying from 1 to 4 weeks. Approximately 4 red blood cell and 5 total white blood cell examinations were made on each animal, together with 5 differential counts, in which a total of 1,000 cells was counted. An average of 4 hemoglobin determinations was made on each of 97 animals, and 4 platelet counts were made on each of 72 animals. The technic of the blood examinations has been described elsewhere (6). The repeated hemocytic observations thus secured were averaged for each rabbit, the absolute numbers of the individual white blood cells being used instead of the relative per cent.

Within 3 weeks after the last blood examination had been made, each rabbit

received a single inoculation into the right testis of 0.3 cc. of normal saline emulsion of Brown-Pearce tumor tissue. Observations on the progress of the tumor growth were made thereafter at weekly or semiweekly intervals. Animals which had not died in the interim were sacrificed at the end of 60 days; necropsy was performed by a systematic plan, described elsewhere (2), by which the body was divided into 50 areas. Transplantation was said to have been successful if a nodule appeared at the site of inoculation and persisted for 30 days or more, or if tumor tissue were found anywhere in the animal at necropsy.

The individual averages for each blood factor were compared with standard values available from a previous study of repeated and averaged similar determinations in 180 male rabbits of the same age, representing 15 standard breeds, in which such variables as sex, season, time of examination, technical errors, food, housing, and disease were eliminated or held constant (6). The means and standard deviations secured in the earlier study were used in the present study, the normal limits for any single factor level being defined as those points 1.959964 times the standard deviation above or below the standard mean; 95 per cent of normal observations lie between such limits. Because their blood levels were not included within these limits, 79 animals, one or more from each of the 17 sets of experiments, were eliminated from the final analysis. In addition, from the original 195 animals, 7 were discarded because of the development of intercurrent disease, and another was discarded because it was not submitted to necropsy. The 108 animals upon which the reported results are based were therefore selected by a rigid process of elimination.

The success of transplantation was first analyzed by means of the χ^2 test (9, 10, 21), with relation to the number of animals with various pretransplantation blood factor levels above or below the standard means (Table I). The animals were then separated into 2 groups. The first group included those with preinoculation blood factor levels within the intermediate distance above or below the standard mean (standard deviation times 0.67¹), and the second those with levels beyond the intermediate distance above or below the standard means (0.67 to 1.96 standard deviations above or below the standard mean). Each group was composed of approximately 50 per cent of the determinations. Finally, the means and standard deviations of the preinoculation blood factor levels in the groups in which transplantation was successful were compared with those in which it was not.

¹ The standard means and quartile distances employed were, for the various blood cell factors, as follows: 5,370,000, 4,999,000, and 5,741,000 red cells; 69.1, 64.7, and 73.48 per cent hemoglobin; 566,000, 486,000, and 645,600 blood platelets; 7690, 6683, and 8697 white blood cells; 3780, 3038, and 4522 neutrophils per cu. mm., 500, 331, and 669 basophiles per cu. mm.; 111, 50, and 172 eosinophiles per cu. mm., 2550, 1855, and 3245 lymphocytes per cu. mm.; 740, 517, and 963 monocytes per cu. mm.

RESULTS

In 18 of the 108 animals no primary growth appeared and no tumor tissue was found at necropsy. Transplantation was therefore said to have been unsuccessful. In the remaining 90 animals a nodule appeared at the site of inoculation and persisted for 30 days or more, or tumor tissue was found at

TABLE I

Association of Blood Cell Factors with Transplantability of the Brown-Pearce Tumor

Blood factor level		R no.	H no.	P no.	W no.	N no.	B no.	E no.	L no.	M no.
High	S	39	29	24	42	40	50	52	40	38
	U	13	11	3	3	6	8	11	5	8
Low	S	51	55	39	48	50	40	38	50	52
	U	5	2	6	15	12	10	7	13	10
Total		108	97	72	108	108	108	108	108	108
χ^2		5.10	11.660	0.08	5.560	0.75	0.75	0.07	1.71	0.03
P		0.02	-0.001	0.78	0.018	0.40	0.40	0.80	0.20	0.85
Intermediate	S	51	42	30	49	57	43	62	53	52
	U	8	13	4	11	10	12	10	12	13
Extreme	S	39	42	33	41	33	47	28	37	38
	U	10	0	5	7	8	6	8	6	5
Total		108	97	72	108	108	108	108	108	108
χ^2		0.90	11.46	0.00	0.50	0.37	2.14	1.20	0.37	1.32
P		0.38	-0.01	0.99	0.48	0.60	0.15	0.27	0.57	0.25

NOTE: No. = number of rabbits; R = red blood cells; H = hemoglobin (available on 97 animals only); P = blood platelets (available on 72 animals only); W = white blood cells; N = neutrophiles; B = basophiles; E = eosinophiles; L = lymphocytes; M = monocytes; High = mean + 0.0-1.96 standard deviations; Low = mean - 0.0-1.96 standard deviations; Intermediate = mean \pm 0.0-0.67 standard deviations; Extreme = mean \pm 0.67-1.96 standard deviations; S = successful transplantation; U = unsuccessful transplantation; χ^2 (see Fisher's *Statistical Methods for Research Workers*); P = probability.

necropsy. Transplantation was therefore said to have been successful in these animals.

Analysis of the results of the various blood studies showed that the pre-transplantation levels of the blood platelets, neutrophiles, basophiles, eosinophiles, lymphocytes, and monocytes bore no statistically significant relation to the success or failure of transplantation of the Brown-Pearce tumor. Furthermore, the average preinoculation levels for the stated factors were not significantly different in the groups in which transplantation was successful and those in which it failed (Tables I and II).

Transplantation was significantly more unsuccessful among animals with preinoculation red blood cell and hemoglobin levels above the standard mean; among animals with hemoglobin levels within the intermediate distance above and below the mean; and among animals with total white blood cell levels (especially within one standard deviation) below the standard mean. The means and standard deviations of these blood factors were significantly different in the groups in which transplantation was successful and those in which it was not. It was therefore concluded that the red blood cell, white blood cell, and hemoglobin levels were apparently related to the success or failure of transplantation.

TABLE II
Preinoculation Blood Factor Levels among Animals with Successful and Unsuccessful Transplants of the Brown-Pearce Tumor

Transplantation		R	H	P	W	N	B	E	L	M
Successful	<i>n</i>	90	84	63	90	90	90	90	90	90
	<i>Mx</i>	535.26	66.77	537.43	755.33	366.58	55.267	128.78	248.57	71.122
	<i>Sm</i>	5.83	0.713	15	19.0	11.65	2.64	9.4	10.91	3.5
Unsuccessful	<i>n</i>	18	13	9	18	18	18	18	18	18
	<i>Mx</i>	560.11	70.31	548.00	685.33	333.83	49.11	131.67	219.00	67.55
	<i>Sm</i>	101	0.54	39.9	20.92	16.80	4.35	21.82	16.1	8.0
Difference	<i>d</i>	24.85	3.54	10.6	70.00	32.75	6.16	2.89	29.57	3.57
	<i>Sd</i>	±11.63	±0.90	±42.6	±28.24	±20.45	±5.10	±23.8	±19.50	±8.73
	<i>t</i>	2.13	3.9	0.248	2.48	1.6	1.21	0.121	1.516	0.409
	<i>n</i>	106	95	70	106	106	106	106	106	106
	<i>P</i>	0.04	0.001	.	0.013

NOTE: R = red cells, H = hemoglobin, P = platelets, W = total white cells, N = neutrophils, B = basophiles, E = eosinophiles, L = lymphocytes, M = monocytes; *n* = number of observations; *Mx* = mean; *Sm* = standard error of the mean; *d* = difference between the means; *Sd* = standard error of the difference; *t*, *n*, see Fisher (*Statistical Methods for Research Workers*); *P* = probability.

DISCUSSION

The red blood cell and hemoglobin levels in individual animals were significantly correlated ($r = +0.666$, $n = 95$, $P = 0.0001$) (10). The relation of the hemoglobin level to the success of transplantation, although similar to that of the red blood cell level, was much greater. No significant correlation, however, existed between the hemoglobin and total white blood cell levels ($r = +0.175$, $n = 95$, not significant). It was concluded, therefore, that the hemoglobin-red cell (as one) and the total white blood cell levels were the only ones of the 9 preinoculation blood cell factors studied which were independently related to the success of intratesticular transplantation of the Brown-Pearce tumor.

In a preliminary publication based on studies of the preinoculation blood factor levels (including 35 animals of the present 108) it was concluded that

deviation of certain pretransplantation levels above or below the species mode for a given factor might be related to the subsequent susceptibility of the animals to intratesticular transplantation with the Brown-Pearce tumor (4). Since that report was made it has become evident that the reaction of the rabbit to this tumor should be considered in two phases, namely the success of transplantation, and the later spread of the neoplasm in the body of the host.

The paradoxical relationship of susceptibility to modal deviation apparent in the preliminary study was interpreted by Strong² as indicating that many of the animals used were diseased, therefore had abnormal blood factor levels, and therefore exhibited increased susceptibility to transplantation (26). The same criticism cannot be made of the present study, since by the rigorous selection practiced, 79 of an original 187 animals, although apparently healthy, were discarded because they presented one or more abnormal blood factor levels. These rejections represented 42 per cent of an apparently healthy population.

With these variables thus controlled, the possible relationship of marked deviation from the normal as the underlying factor in the success of transplantation of the tumor was investigated in the 108 rigidly selected animals.

1. The distributions of the preinoculation hemoglobin levels (Fig. 1) and total white blood cells (Fig. 2) were plotted in per cent and the modal points noted. The mode for the total white blood cells was 7,000 per cu. mm., and there was a skew to the right. The mode for the hemoglobin was 70.5 per cent, and there was a skew to the left. Both distribution curves were identical within the error of sampling with standard curves for the factors, and the modes were identified with the standard modes (Figs. 1 and 2).

2. The percentage of animals resistant to transplantation was plotted for each preinoculation level and for each factor (Figs. 1 and 2).

The groupings about the mode illustrate graphically the statistically significant relations of the white blood cell and hemoglobin levels to the success of transplantation, the mode and mean of the total blood cell levels being more widely separated from each other than the mode and the mean of the hemoglobin levels. The paradoxical relation of susceptibility to deviations from the mode was more striking in this study than in the preliminary study. In fact, the distribution and the resistance curves were identical except for the skewed portions. Skew values for the hemoglobin-red cell towards anemia and for the white cell values towards leukocytosis were associated with success of transplantation.

When the 79 animals discarded because of abnormal preinoculation blood values were analyzed, no statistically significant relation of the hemoglobin,

² Strong's observation that mice of cancer strains developed anemia before the gross demonstration of malignant changes did not settle the problem of which was cause and effect.

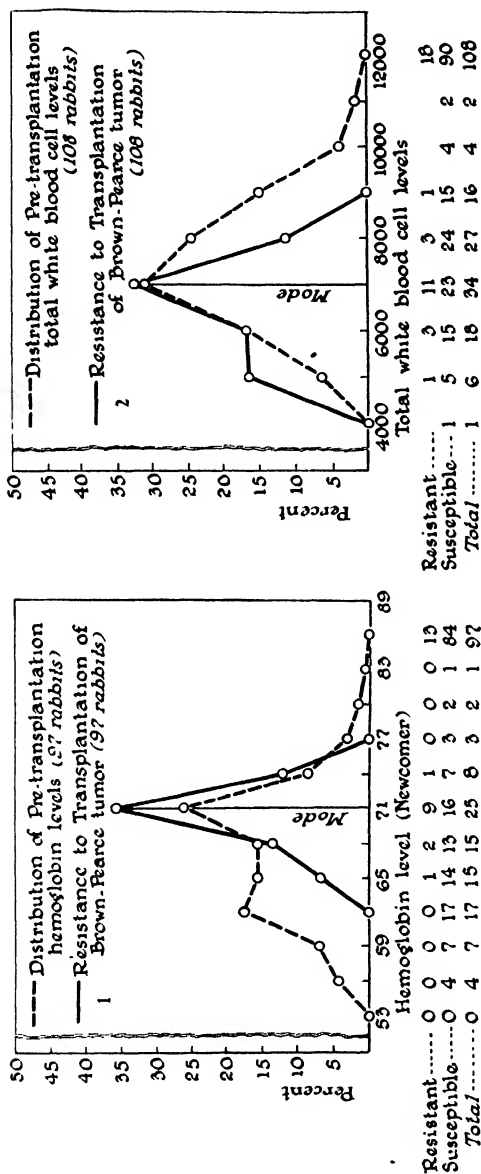


FIG. 1

FIG. 1. Paradoxical relation of the distributions in per cent of the hemoglobin level and the transplantability of the Brown-Pearce tumor in normal rabbits. Note the discrepancy for skew values.

FIG. 2. Paradoxical relation of the distributions in per cent of the total white blood cell level and the transplantability of the Brown-Pearce tumor in normal rabbits. Note the discrepancy for skew values.

FIG. 2

the red blood cell, and the total white cell levels to the success or failure of transplantation could be demonstrated although the trends were in the same directions. The importance of a normal blood formula in experimental work where a standard response is expected is thus demonstrated.

Although the absolute numbers of the various white blood cells were used in the study the levels in relative per cent were calculated for the neutrophils, basophiles, eosinophiles, lymphocytes, and monocytes. The levels in relative per cent were analyzed through the use of correlation tables, high and low, and intermediate and extreme groupings. No statistically significant relationship of the levels in relative per cent of the success of transplantation of the Brown-Pearce tumor was obtained.

All resistant animals had hemoglobin levels within 0.67 times the standard deviation above or below the mode for normal rabbits, and all but one had total white blood cell levels within this range. The single exception was within the limits of error from the modal group. Every animal with hemoglobin or white blood cell levels or both above or below these limits was successfully transplanted.

For each character approximately half the observations fell within ± 0.67 times the standard deviation of the mode. Since the total white blood cell and hemoglobin levels are independent variables, it would be expected that one quarter of the 97 animals upon which these values were available would have both levels within this distance of the mode. Actually, 25 animals fell into this group. If resistance to the intratesticular transplantation of the Brown-Pearce tumor were the summation effect of three independent variables with similar frequency distributions and with levels within ± 0.67 standard deviations of the mode, it would be expected that 12.125 animals would be resistant to the neoplasm. Actually, there were 13 resistant animals. These observations strongly suggest that a third unknown variable or variables, together with the hemoglobin-red cell and total white blood cell factors, would fully explain the resistance of the rabbit to intratesticular transplantation of the tumor by the methods employed.

A search among other blood factors studied and the construction of correlation tables of various sorts failed to reveal any possible linear or alinear relation other than that of the hemoglobin-red cell and total white blood cells. The small number of observations on the blood platelets unfortunately did not permit adequate analysis. No relationship of the red blood cell level to the success of transplantation could be demonstrated when the hemoglobin and total white blood cell levels were held constant. The total white blood cell value was found to be the summation effect of each of the constituent white blood cell elements (which were insignificant in themselves) and not of one or two elements individually. This observation suggests that factors affecting blood volume were responsible for the relationship of the total white blood

cells to the success of transplantation rather than the white blood cells *per se*. Variations in the plasma protein levels are a possibility (27).

The distance mode ± 0.67 standard deviations was selected arbitrarily and was employed only because it divided the data into two approximately equal parts; it would be quite possible to employ some other division. The fact that quantitative data such as blood cell levels can be altered by environment as well as by inheritance suggests a basis for the study of the relative effects of heredity and environment. The importance of blood factor level variations such as these emphasizes the danger of limiting observations to a single inbred strain of animals or to too few standard strains in the study of factors involved in mammalian resistance.

SUMMARY AND CONCLUSIONS

In order to determine whether the transplantability, growth, and spread of the Brown-Pearce tumor might be influenced by factors reflected in the pretransplantation blood formulae, seventeen sets of experiments were carried out upon 195 young adult male rabbits, in apparent good health. Repeated determinations were made of the levels of the red blood cells, hemoglobin, platelets, total white blood cells, neutrophils, basophiles, eosinophiles, and monocytes. Seventy-nine animals were discarded for no other reason than that one or more average blood factor levels were abnormal, and 8 animals were discarded for other reasons. The final analysis was therefore carried out on 108 animals. After the blood examinations had been concluded, the animals were inoculated intratesticularly with the Brown-Pearce tumor. Transplantation was unsuccessful, by the criteria set up, in 18 animals.

No relationship could be demonstrated between the success or failure of transplantation and the pretransplantation levels of the blood platelets, neutrophils, basophiles, eosinophiles, monocytes, or lymphocytes. A statistically significant relationship was, however, demonstrated between the success and failure of transplantation and the pretransplantation levels of the hemoglobin, the red blood cells, and the total white blood cells. The hemoglobin and red cell levels were interrelated and the relationship of the red blood cell level to the success of transplantation seemed secondary to the hemoglobin level. No relationship of the red blood cell level to the success of transplantation could be demonstrated when the hemoglobin and total white blood cell levels were held constant.

The resistance of the rabbit to transplantation of the Brown-Pearce tumor was found to be associated with optimal or modal pretransplantation levels of the hemoglobin and the total white blood cells. When the average level of either the hemoglobin or the total white cells (independent variables) was not modal the animals were susceptible. Besides the hemoglobin and total white cell levels, the presence of a third unknown or unidentified factor (or factors) was postulated.

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THE SPINAL MECHANISM OF THE PYRAMIDAL SYSTEM IN CATS*

By DAVID P. C. LLOYD

(From the Laboratories of The Rockefeller Institute for Medical Research)

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It is the purpose of this study to examine the functional organization of that portion of the spinal integrating mechanism which is subject to activation by the pyramidal system.† To this end it is imperative that other sites of integration be eliminated, and that pyramidal impulses alone be allowed to enter the spinal cord. Stimulation of the "motor" cortex may not be employed, because cortical facilitation will result (Graham Brown, 1915a; Adrian, 1936), and because of the inevitable participation of the extra-pyramidal system in the distribution of activity to the spinal cord (Schäfer, 1910; Tower, 1936). Just as complete interruption of the pyramidal tracts without damage to other systems may only be accomplished by section of the pyramids in the medulla (Marshall, 1936), so only at the pyramids may the tract be stimulated without activation of other systems, and then only by the use of additional precautions. Other elements within the medulla, notably the reticular formation, may be activated by pyramidal collaterals or by direct extension of the stimulating current. The latter mode of activation has been known to occur even when electrodes designed to concentrate the stimulating current across the pyramids have been used. It has been necessary, therefore, to interrupt all pathways other than the pyramids at a level caudal to that of the stimulating electrodes. In consequence the preparations are essentially spinal, for conversion from the decerebrate state to the spinal state, in the cat, accompanies lesions of the vestibular nucleus or its direct pathway (Fulton, Liddell, and Rioch, 1930a, b).

It is not enough that pathways other than the pyramidal tract be interrupted below the site of the stimulating electrodes. A complete transection cranially is necessary to prevent ascending activity from reaching the cortex by various channels (antidromically along the pyramidal fibers, thence by collaterals, or dromically through the medial lemniscus and thalamus, etc.), there to activate pyramidal neurons. With all the foregoing precautions, the necessity for which

* A preliminary account of some of the present results was presented at the meeting in Chicago of the American Physiological Society (Lloyd, 1941c).

† The definition of the pyramidal tract used herein follows from the descriptions of Türck (1851, 1853) and Flechsig (1876), *i.e.* the pyramidal tract consists of those fibers which pass through the pyramid to the spinal cord. For a discussion of the use of the term pyramidal tract cf. Marshall (1936).

has been shown by experience, the activity reaching the spinal cord as a result of a single pyramidal stimulus is a controlled single pyramidal volley.

Cats have been used throughout the present investigation. These were lightly narcotized with Dial or nembutal. Supplemental ether was regularly used until the preparation was decerebrated or rendered essentially spinal. Most of the preparations made in this way were at the upper limit of irritability beyond which the present type of experimentation is not practical, even with the spinal cord held in position by a system of spinal clamps. The slightest movement of the nervous tissue under observation is inimical to the successful recording of "spike-like" activity with rigid microelectrodes. To describe

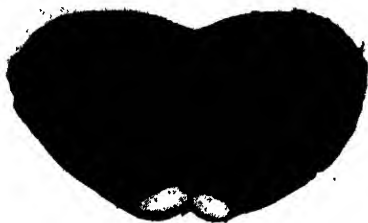


FIG. 1. Gross specimen to illustrate the standard lesion placed at the level of the inferior olives by means of a spring guillotine. The medulla was removed at the end of the experiment and fixed. After fixation the specimen was rapidly surface-stained, washed and blotted. The lesion was completed and the cut face of the transected medulla photographed. The unstained area shows the extent of the cross-sectional area that is spared by the guillotine, and hence the maximum conducting tissue between the stimulating electrodes and the spinal cord.

further the functional state of these preparations, it may be noted that they usually exhibited a strong tonic motoneuron discharge (Fig. 12A), which in the absence of vestibular background was probably maintained by the constant influx of afferent impulses from the periphery (Barron and Matthews, 1935). A record of this tonic afferent activity will be presented in another connection (Lloyd, to be published). It is probable, in the light of recent studies (Marshall, Woolsey, and Bard, 1941; Lloyd, 1941a) that the low concentration of barbiturate present in these preparations (some were observed as long as 14 hours after the administration of nembutal in amounts sufficient for initial basal narcosis) did not interfere appreciably with primary responses to pyramidal stimulation.

Stimulating electrodes were placed through the floor of the fourth ventricle until the bared tips embraced the pyramid at the level of the trapezoid body.

Cranially a complete transection was placed at the collicular level. The lesion caudal to the electrodes was accomplished by means of a spring guillotine. As the blade of this guillotine passes ventrally through the medulla until the curved free border reaches the basiocciput, a central notch in the blade permits the pyramids and basilar artery to escape damage. Figure 1 illustrates, in a macroscopic preparation, the extent of the standard lesion produced by the guillotine at the level of the inferior olives. The medulla was removed from each preparation at the end of the experiment, and the lesion inspected after fixation.

Records were obtained by means of fine steel wire microelectrodes insulated but for the tip. These could be placed at will in the spinal cord with the aid of a micromanipulator, used with due regard for deformation error. Approach to regions of the gray substance from various angles in many experiments has diminished the hazard inherent in mechanical error. The highest degree of accuracy cannot be claimed in the absence of histological control; however, the latitude in localization which has been allowed for in the responses to be described is ample to compensate for the errors involved.

Conduction in the pyramidal tract. A single shock to the pyramid results in a volley of impulses which is conducted caudally throughout the length of the spinal cord. The response of the pyramidal fibers may be recorded from a highly localized area equal to approximately 1 sq. mm. of the cross-sectional area of the cord, and situated as expected in the cat, in the lateral column adjacent to the dorsal horn (cf. Spitzka, 1886; Lenhossék, 1889; Bechterew, 1890). Figure 2 illustrates the pyramidal tract response to single shocks recorded at various conduction distances. One would expect the pyramidal fibers conducting impulses past the recording microelectrode to yield impulses recordable as triphasic spike potentials (for a recent discussion cf. Lorente de Nó, 1939, p. 430 *et seq.*). An attempt to obtain such records even at short conduction distances has been only partially successful. For example, Fig. 2A shows a small negative deflection recorded at a conduction distance of 6.8 cm. If the recording microelectrode is thrust boldly into the region occupied by the pyramidal fibers, or having passed through that region is brought back along its own track, a large, predominantly positive potential results. Figure 2B which shows such a positive potential, was recorded from the same point as was Fig. 2A. Similar effects have been noted by Therman (1941) when attempting to record from the medial lemniscus, and by Bishop and O'Leary (1941) in the superior colliculus. The origin of the predominantly positive responses, such as are shown in Fig. 2, is not entirely clear. Part at least of the initial positive deflection may be ascribed to the impulses approaching from a distance. Consequently it is difficult to designate exactly the time of arrival of impulses at the recording electrode from such records. Damage is an undoubted factor, but some of the recorded impulses reach or pass the electrode,

for there are spike potentials in the records having a prominent negative phase, although they are submerged by positivity. The resultant records probably indicate a dispersed discharge in a number of elements, some of which are

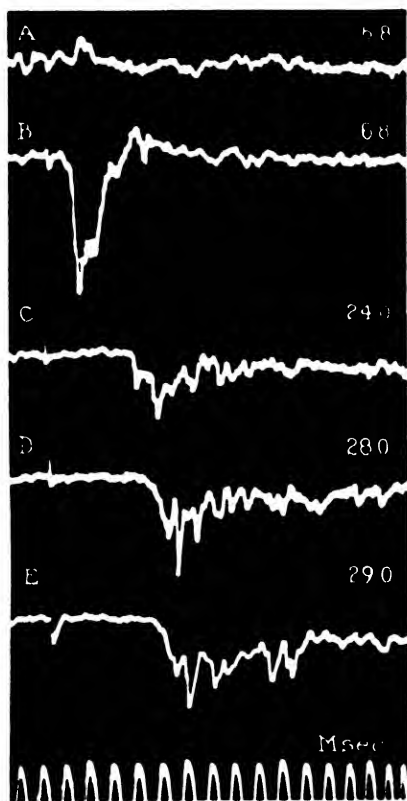


FIG. 2. Activity instituted by single pyramidal volleys and recorded by micro-electrode from the pyramidal tract at the conduction distances designated in centimeters at the right. A to D from one preparation. E from another preparation for comparison. Time in msec. Further description in text.

blocked at a short distance from the electrode, some of which are blocked at the electrode, and some of which are unblocked.

Records 2C, D, and E were obtained in the same way as was 2B, but at the designated conduction distances. Records 2A to D were obtained from a single preparation. Record 2E is from another preparation for the purpose of comparison. A slope of the latencies in 2B to D reveals a conduction rate of approximately 63.5 M per sec. for the most rapidly conducting fibers of the tract. This value was approximated in all the experiments of this series.

Determination of the lower limit of pyramidal fiber velocities is fraught with

uncertainty. The activity as recorded from the tract has no clearly defined termination. The tract itself has intimate anatomical relationship with cellular elements which are known to discharge in response to the tract impulses (cf. next section). Thus, although the pyramidal response certainly suffers considerable temporal dispersion, no definitive value may be given for the lower limit of velocities. A study of the records suggests that an estimate of 18 M per sec. is not too low for the lower limit of velocities, although in view of the large numbers of very fine fibers to be found in the pyramidal tract of the cat (McKibben, and Wheelis, 1932; Lassek and Rasmussen, 1940), this value may be far too high.

The activity illustrated in Fig. 2 is the only tract activity that has been recorded from the white columns of the spinal cord following pyramidal stimulation, as would be expected from a consideration of the anatomical studies of Lenhossék (1889) and Bechterew (1890) on the cat's spinal cord.

It is important to recognize the implications of the fact that the pyramidal tract, subsequent to synchronous activation at the level of the medulla, delivers into the lumbar enlargement, by virtue of dispersion, a prolonged asynchronous discharge. In consequence some features of the tract activity will resemble those of a diffuse nuclear discharge. The major principle is: that time lost by slow conduction is in some measure the formal equivalent of time lost in the synaptic relaying of otherwise rapidly conducting pathways. For example, it will be seen that the most rapidly conducted impulses evoked by a second pyramidal shock will be capable of summation with the more slowly conducted impulses evoked by an antecedent similar shock, given an interval of several milliseconds between shocks and a similar distribution of terminal knobs.

Responses of the Gray Substance

(1) *The external basilar region (Cajal).* Following a single shock to the pyramid, and the consequent arrival of a dispersed volley of pyramidal impulses in the lumbar enlargement, certain interneurons within the cord become active. The resulting activity is discernible often only as a slow potential change of some 40 to 45 msec. duration. Under favorable conditions however, a burst of small spike potentials occupying the period of the slow potential wave is recorded. An experiment of this kind is presented in Fig. 3. Record 3A shows the level of noise and activity maintained in the absence of any specific stimulation. The recording tip of the microelectrode was placed in the extreme lateral margin of the base of the dorsal horn (external basilar region, Cajal, 1909). The anatomical position is confirmed by the appearance in the record (3B) obtained with a single pyramidal volley, of both pyramidal impulses (p) and nuclear discharges (e). The pyramidal impulses recorded in Fig. 3 are probably occupying pyramidal collaterals to the gray substance.

The pyramidal impulses begin after a latency of approximately 4 msec., are again of positive sign (indicating damaged bundles of fibers), and are accom-

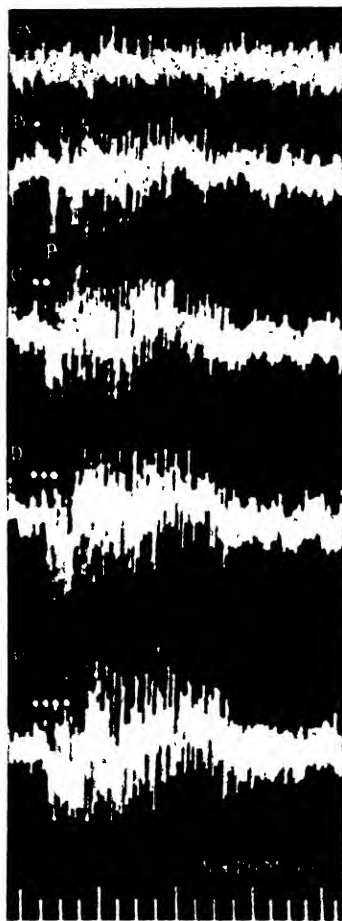


FIG. 3. Activity recorded at the extreme lateral aspect of the base of the dorsal horn. The stimulation artifacts of the pyramidal shocks are marked by dots. Pyramidal impulses and nuclear discharges are identified in B (p and c respectively). Time, in 5 and 20 msec. intervals, is shown at the bottom. In all figures where there are two time designations, these are for the small and large divisions respectively. Further description in text.

panied almost immediately by a flame of small spike potentials, the latter making up a discharge not greater than $50\mu\text{V}$. in amplitude with a duration of some 40 msec. It seems certain, by reason of the fine and obviously non-unitary character of the nuclear discharge, that it may be assigned to a dense

population of small elements situated at the lateral aspect of the base of the dorsal horn, and intermingled with fascicles of pyramidal collaterals.

The close functional relationship between the pyramidal tract and nuclear elements at the base of the dorsal horn forces the opinion that the pyramidal fibers impinge, either terminally or collaterally, upon these elements. This interpretation agrees best with the view held by Schäfer (1899), who stated that the pyramidal fibers enter the gray substance at the base of the dorsal horn and end in relation to cells of that region. Other zones of termination are not excluded; for instance, although the type of discharge at present under consideration appears to be more prominent at the external basilar region of Cajal, it is probable that small elements reacting to pyramidal stimulation in a similar manner are more widespread. A suggestion of low amplitude discharges is to be found in other regions following single shocks to the pyramids (Fig. 5B, 6A and G). It is difficult to affirm that these discharges in other regions result clearly, from the pyramidal stimulation. Hence it would be gratuitous to assume, on this basis alone, a more widespread distribution of pyramidal fibers, although some histological studies on the cat do point to this direction (Hoff, 1932).

Records 3C, D, and E show the manner in which the fine basilar discharge is intensified by the addition of successively greater numbers of pyramidal shocks. It is only after a degree of intensification approximating that seen in Fig. 3D and E, that other types of activity have been found in other regions of the gray substance, *i.e.* intensification of activity in one region is accompanied or followed by spread of activity to other regions. The active regions, subsequent to spread, include the solitary cell region of the dorsal horn and the intermediate region.

(2) *Solitary cells (Lenhossék) of the dorsal horn.* The responses obtained in this region have all the characteristics that would be expected from a group of large cells sparsely scattered through a "matrix" of smaller elements. The correlation between functional picture and histological description (Lenhossék, 1895; Cajal, 1909; Bok, 1928, etc.) appears to justify the argument that the discharges under consideration are recorded from the solitary cells of the dorsal horn. The responses are localized, and when found, are relatively uniform in amplitude and fairly regular in frequency when subjected to a prolonged pyramidal tetanus (Fig. 4). At no time in this series of experiments have two similar units of the solitary cell type been detected at the same microelectrode position. On the basis of general experience this fact would indicate that the units are few in number and widely spaced. In general, all the criteria upon which the assumption of unitary response is customarily based (*cf.* Adrian and Bronk, 1928, etc.) are satisfied. The amplitude of the spike potentials recorded from these units is large, being usually of the order of 2mV. It may be stated parenthetically that the only larger spike potentials (up to 6mV.) which

have been encountered in the spinal gray substance so far are those attributable to motoneurons.

Figure 4 illustrates the characteristic discharge of a solitary cell unit of the dorsal horn when driven by pyramidal tetani of increasing durations. As seen

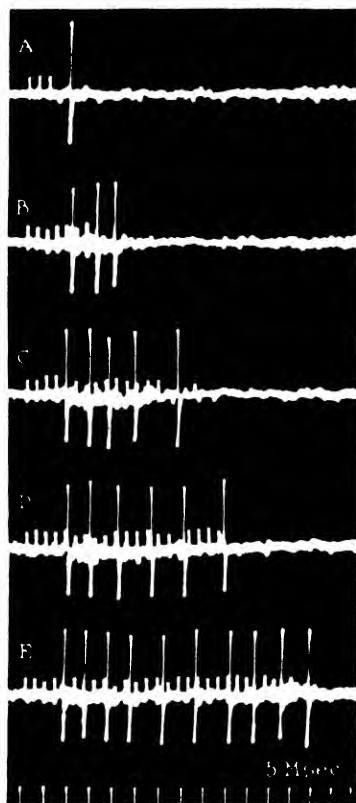


FIG. 4. Activity found in the center and base of the dorsal horn. The small regular deflections are the recorded stimulus artifacts of the pyramidal shocks. The large spike potentials (about 2 mV.) constitute the solitary cell response to pyramidal stimulation.

in Fig. 4A, three pyramidal shocks are necessary to produce the first and single response of the unit. As the tetanus is lengthened (4B to 4E) subsequent discharges occur. A glance at the records of Fig. 4 suffices to show that there is a roughly direct relationship between the duration of the pyramidal tetanus and the number of discharges obtained from this unit. Even so the discharge frequency bears no direct and exact relationship to the stimulus frequency.

The solitary cell discharge takes place on a background of small cell activity that may be identified in the observations of Fig. 4. It is probable that the

highly asynchronous activity of the small units in this region, as well as in the external basilar region, constitutes a slowly changing, statistically smooth over-

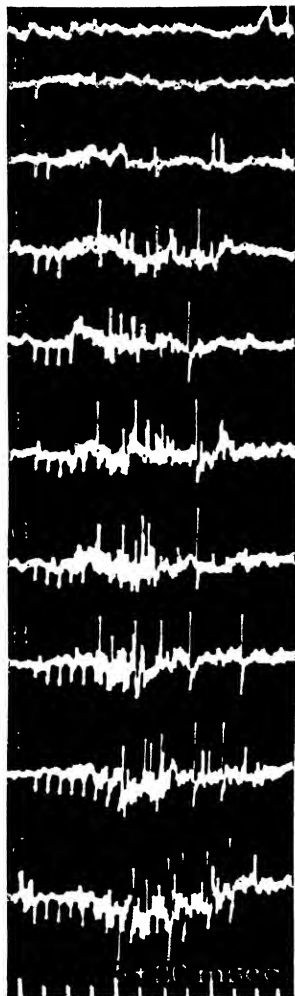


FIG. 5. Activity in the intermediate region resulting from one (B) to nine (J) pyramidal shocks. The level of "resting" activity is shown in A. Further details in text.

all excitation to the large solitary cell units, and that these latter in turn respond at intervals determined in part by their own properties (cf. Lorente de N6, 1938, p. 226). The facts, (i) that the solitary cell units discharge only after a 9 or 10 msec. total latent period, (ii) that summation of influences from several pyramidal shocks is necessary to procure a discharge, and (iii) that the

stimulus frequency is not evident in the response (cf. with Fig. 7) suggest that the internuncial contribution to the solitary cells is of relatively greater importance than is that of the pyramidal fibers themselves. It is not possible to exclude pyramidal fibers from synaptic relationship with the solitary cells. Whether or not the solitary cells represent important relays distributing pyramidal activity of the motoneurons of the same side depends naturally upon the distribution of the solitary cell axons. Some of these cells have axons passing through the ventral commissure to the opposite side, some have uncrossed axons reaching to the lateral columns (Cajal, 1909; Bok, 1928). The latter might be regarded as reaching the motoneurons of the same side. It is furthermore possible, on the basis of time relationships, that the solitary cells relay activity to the intermediate region.

(3) *The intermediate region, including the intermediate gray nucleus of Cajal.* Units throughout the intermediate region discharge impulses in response to a short train of shocks to the pyramid. The spike potentials yielded by these units have an amplitude of $200\mu\text{V.}$ to 1 mV., in general, intermediate in amplitude between the spike potentials of the two groups already considered. The individual units of the intermediate region are not as discrete as are the solitary cells, as evidenced by the fact that even the most favorable records usually contain spike potentials from several similar elements. The intermediate response is most prominent in the ventral part of the intermediate region.

The latent period for activity of the intermediate type varies usually between 12 and 20 msec. in duration. The method of direct recording from a few units is not the most satisfactory method for observing the average discharge latency of a neuron pool, since quite wide variations often occur between successive observations. The values given are thus of necessity estimates based upon a large number of observations in a number of experiments. The frequency of pyramidal stimulation is a factor influencing the latency of response in the intermediate region, as elsewhere. Various examples to illustrate the latency for discharge in the intermediate region may be found in Figs. 5 and 6.

Figure 5 demonstrates the effect of applying successively longer trains of pyramidal shocks upon the activity in the intermediate region. The micro-electrode in this experiment was placed 2.0 mm. below the dorsal surface of the cord, just medial to the root entry line. A comparison of record 5A, in which no stimulus was given, with 5B, in which a single pyramidal shock was delivered, indicates clearly that a single pyramidal volley does not precipitate activity of the intermediate type. It is only in record 5D, obtained with the use of three pyramidal shocks, and in the subsequent records of Fig. 5, that unmistakable activity of intermediate type is to be found. The fact that several pyramidal shocks are necessary to bring about the intermediate type activity has been regularly and repeatedly confirmed. Other examples of this observation may be found in Fig. 6A to F, recorded again from the lumbar enlargement,

and in Fig. 6G to L, recorded from a similar region in the cervical enlargement. It is of particular interest that several pyramidal volleys are required to obtain an intermediate type discharge in the cervical cord, for there dispersion of the pyramidal tract volleys is so much less (compare Fig. 2B with Fig. 2C, D, and E). It may be inferred that dispersion of the pyramidal impulses *per se* is not

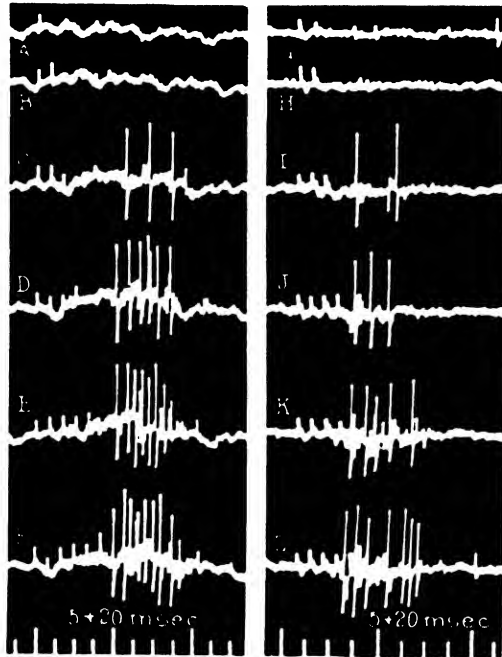


FIG. 6. Activity in the intermediate region resulting from one to six pyramidal shocks. A-F recorded from the lumbar cord. G-L recorded from the cervical cord. Note the difference in total latency for activation of the intermediate regions of cervical and lumbar enlargements under otherwise similar conditions, also background discharges of low amplitude.

the primary cause of the failure of the intermediate region to respond to single pyramidal volleys.

A comparison of the activity characteristic of the intermediate region with that most prominent at the external basilar region (Fig. 3) shows that the former only occurs under conditions which are known to intensify the latter, and furthermore, that the former begins later and ends earlier than the latter under similar durations of pyramidal stimulation. A study of Figs. 5 and 6 reveals that the intermediate type activity appears on a background of fine discharges which are in no fundamental sense different from those in the external basilar region. The relationship between small cell activity (external

basilar and intermediate) and the intermediate type activity is such as to lead to the conclusion that the intermediate type units become active largely as the result of discharges projected from the small cell elements. This conclusion does not exclude the possibility of direct synaptic connections from pyramidal

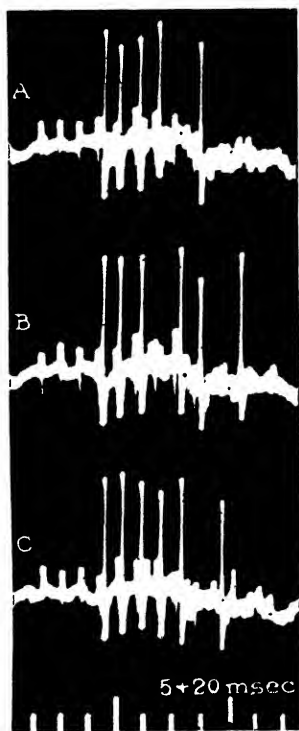


FIG. 7. Example of nuclear elements of the intermediate region following exactly the pyramidal stimulus frequency. As the frequency is high, a fairly close relationship between tract fibers and these nuclear elements is indicated.

fibers. Connections of the latter sort must in fact be considered for the following reason (cf. Fig. 7):

In the responses presented so far there has been no indication that units of the gray substance follow the frequency of the pyramidal stimulus. This may not be cause for surprise, since dispersion is great and the stimulation frequency high (cf. Bronk, Pitts, and Larrabee, 1940). Figure 7, however, shows a single experiment in which a unit was found to follow exactly the stimulation frequency. The microelectrode was placed in the intermediate region (2.1 mm. down, 0.5 mm. medial to the root entry line). In each record of Fig. 7 (all similar) some of the responses are dropped. The dropped responses are

the fifth, seventh, and eighth in A; the fourth and seventh in B; and the sixth and eighth in C. Discounting the dropped responses, the remainder are absolutely fixed with respect to the causal pyramidal shocks. The facts (i) that shortening of the pyramidal pathway to this unit does not take place (cf. discussion in connection with Fig. 11), and (ii) that some elements of the intermediate gray substance can be driven at the (high) frequency of the pyramidal volleys indicate a rather close anatomical connection between pyramidal fibers and such intermediate elements.

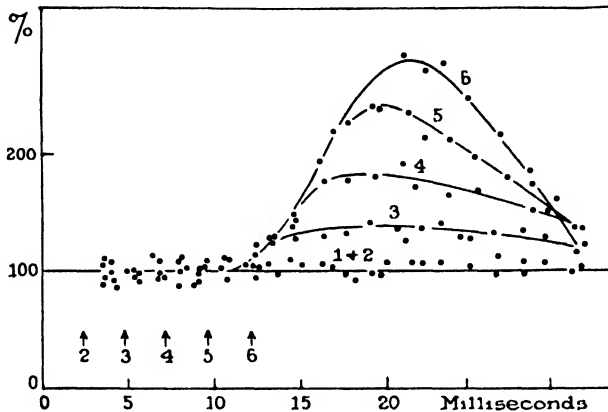


FIG. 8. Facilitation of spinal motoneurons by pyramidal activity. Amplitude of the two-neuron reflex response (ordinates) is plotted against the interval at which the reflex follows the single pyramidal shock, or first pyramidal shock of a train. The numbered arrows indicate the timing of the pyramidal shocks. The number of each curve of the family identifies that curve with the number of pyramidal shocks employed in the conditioning stimulation. Amplitude of the test response in isolation is 100.

Facilitation of motoneurons by pyramidal action. The two-neuron-arc reflex discharge described by Renshaw (1940) has proved of value for the purpose of testing the average excitability of a segmental population of motoneurons. Since the motoneuron discharge that results from short trains of pyramidal shocks cannot be measured with any degree of accuracy, it has been necessary to rely upon alterations in testing two-neuron-arc reflex discharges for a reliable gauge of pyramidal influence over the motoneurons.

Figure 8 shows plotted curves constructed from an experiment in which the influence, on the two-neuron-arc discharge, of one to six pyramidal shocks was examined. The coordinates show the amplitude of the two-neuron-arc discharge as a function of time after the onset of the pyramidal stimulation. A single or two pyramidal shocks (curves 1 and 2) had no effect on the motoneurons that could be detected by the method. Three shocks resulted in a

brief period of facilitation in the motor pool, after a total latent period of approximately 12 msec. The total latent period for facilitation at the motoneuron level varies from 12 to 20 or more msec., its duration being, in part, a function of the frequency of pyramidal stimulation (compare the motoneuron facilitation curves of Fig. 9A and B). Figures 9, 10, and 11 show examples from other experiments of the total latency for motoneuron facilitation. The values found in these experiments were approximately 14 and 18, 15.5, and 12 msec. respectively.

As more pyramidal shocks are added to the train, facilitation of the motoneurons increases in intensity (Fig. 8, curves 4, 5, and 6). The duration of the facilitation period due specifically to the sixth pyramidal shock (obtained by subtracting the area enclosed by curve 5 from that enclosed by curve 6) is much shorter than is the duration of the facilitation period specifically related to the third shock (area enclosed by curve 3). Thus, to parallel the increase in intensity there is a more rapid summation of subnormality (Gasser, 1935; Lorente de Nó and Graham, 1938) that occurs at the pre-motoneuron level rather than at the motoneuron level itself, for the feeble motoneuron discharge is not likely to evoke significant motoneuron subnormality. On this interpretation, the more rapid failure of pre-motoneuron activity consequent upon more intense activation would increase reflex threshold in the ventral horn secondarily by diminished opportunity for summation there.

A comparison of facilitation in the motor nucleus (Fig. 8, also Figs. 9, 10, and 11) with discharges recorded from the intermediate region of the gray substance (Figs. 5 and 6) shows that the two events are closely parallel. Given anatomical connection (Kölliker, 1890; Cajal, 1909; and others), the parallelism appears to establish a causal relationship. The conclusion may be reached that activity in the intermediate region, following pyramidal stimulation, is the principal contributing factor to excitation in the motoneuron pool. The solitary cells of the dorsal horn, as stated above, may form additional links between the pyramidal fibers and the motoneurons.

Facilitation of reflex arcs at internuncial levels. Just as the two-neuron-arc discharges have proved of use for testing the excitability of motoneurons three-neuron-arc reflex discharges now prove to be equally useful in detecting average excitability changes at the internuncial level, when evaluated in terms of alterations in discharges mediated through arcs of two neurons. In theory, three-neuron-arc reflex discharges may be facilitated at two points; at the junction of primary afferent neurons with interneurons, and at the junction of interneurons with motoneurons. If, however, a three-neuron-arc discharge be facilitated in the absence of any change in the concomitant two-neuron-arc discharge, it may be said that the facilitation has occurred at the internuncial level.

Figure 9 illustrates the effect of pyramidal stimulation on reflex discharges

pertaining to arcs of two and three neurons. The inset B of Fig. 9 shows the dorsal to ventral root reflex discharge resulting from a single shock. The first two clearly defined spike potentials, separated in time by approximately 0.7 to 0.8 msec., are the two and three-neuron-arc discharge spike potentials respectively. It is the amplitudes of these two spike potentials that are

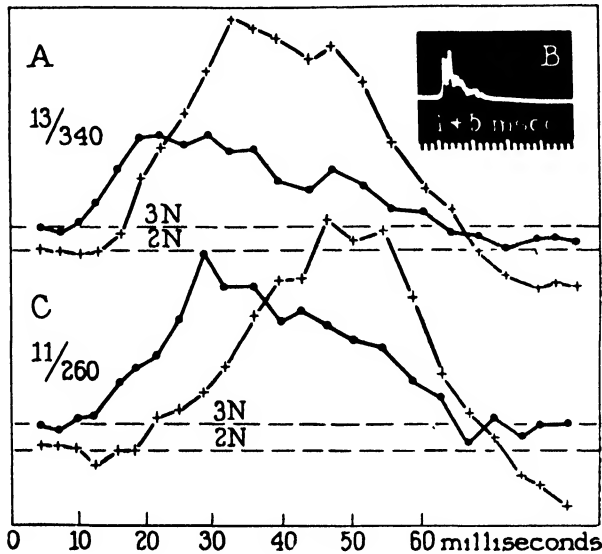


FIG. 9. Facilitation of two-neuron and three-neuron reflex arcs by pyramidal activity. Inset B shows the reflex discharge in response to the test dorsal root volley. The first two spike potentials of B represent activity through arcs of two and three neurons respectively. The amplitudes of the two-neuron response (crosses) and three-neuron response (circles) are plotted in A and C as functions of time after the first shock in a train of pyramidal shocks. The hatched lines, 3N and 2N, of A and C show the amplitude of the test responses in isolation. In A, 13 shocks at 340 per sec. provided the conditioning activity. In C, 11 shocks at 260 per sec. were used for conditioning. Full description in text.

plotted in Fig. 9A and C, as a function of time after the onset of the pyramidal stimulation. In Fig. 9A, 13 shocks were delivered to the pyramid at a frequency of 340 per sec.

Facilitation of the two-neuron-arc response in Fig. 9A (crosses) begins approximately 14 msec. after the onset of the pyramidal stimulation. In contrast, facilitation of the three-neuron-arc reflex discharge (circles) begins several milliseconds earlier, at about 9 msec. after the first pyramidal shock. Thus for a period of approximately 5 msec. the three-neuron-arc may be regarded as being facilitated only at the internuncial (pre-motoneuron) level.

A time differential amounting to as little as 3 msec. between the facilitation of three-neuron and two-neuron-arc discharges has been encountered. The total latency for facilitation of three-neuron-arc discharges varies between 9 and 12 msec. Because the beginning of the solitary cell discharge (Fig. 4) falls within the 9 to 12 msec. time range, it is possible that the solitary cells supply impulses by collaterals to the interneurons occupied by the three-neuron-arc discharges.

As the facilitation of two-neuron-arc discharges progresses, there is a secondary decrease in the amplitude of the facilitated three-neuron-arc discharge, which undoubtedly indicates that a strongly facilitated two-neuron-arc discharge occupies more of the motoneurons than would otherwise be at the service of three-neuron arcs, *i.e.* the facilitated two-neuron-arc discharge occludes the succeeding three-neuron-arc discharge (the law of plurality of connections, Lorente de Nó, 1933, 1938).

The view that two and three-neuron-arc discharges have some degree of reciprocal relationship is strengthened by a consideration of the curves presented in Fig. 9C. Figure 9C is similar to Fig. 9A, with the single exception that 11 shocks were delivered to the pyramid at a frequency of 260 per sec. With the slower frequency of stimulation employed in Fig. 9C, motoneuron facilitation (crosses) begins later (about 18 msec. after the first pyramidal shock) and progresses more slowly to reach a later maximum. Facilitation at the internuncial level also begins later and progresses more slowly. However, the added delay in facilitation is greater at the motoneuron level than at the internuncial level, with the result that the three-neuron-arc discharge may develop further before measurable occlusion begins by virtue of facilitated two-neuron-arc discharges.

In some experiments the two-neuron-arc discharge is so completely dominant in the unisegmental reflex that it becomes impossible to analyze events in higher order chains. Conversely, if a bisegmental reflex is substituted for the unisegmental reflex as a test system, an opportunity arises to study the behavior of three-neuron arcs relatively unencumbered by strong two-neuron-arc discharges. Figure 10 presents records from an experiment in which the bisegmental reflex discharge (10B) is conditioned at various time intervals by 6 pyramidal shocks (10A). The earliest two-neuron-arc discharge occurs in 10H. Between H and O of Fig. 10, two-neuron arcs are facilitated, and presumably less direct reflex pathways also can be facilitated at the motoneuron level. Observations D, E, and F show that delayed segments of the reflex discharge are facilitated before the onset of facilitation at the motoneuron level. In 10G, the three-neuron-arc spike potential (still the initial spike potential of the reflex) is just overlapping the beginning of the period during which facilitation is known to occur at the motoneuron level. The two-neuron-arc facilitation is maximal in 10J, but even so it is so small that it does

not interfere measurably with facilitation of the three-neuron-arc discharge. Occlusion, which is a prominent feature in Fig. 9, is not detectable in Fig. 10, in which the two-neuron-arc discharge is small. This fact again points to the multiplicity of connections between primary afferent collaterals and spinal motoneurons.

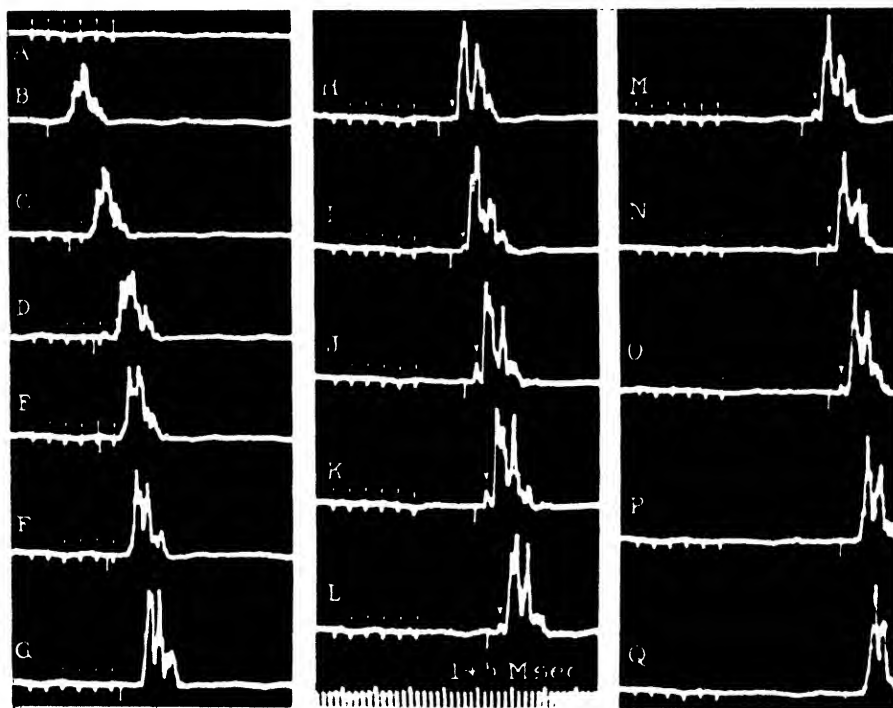


FIG. 10. Effect of six pyramidal shocks on bisegmental reflex discharge (L7-S1). Recording leads on S1 ventral root. A, response to six pyramidal shocks. B, response to testing L7 dorsal root shock. In C to Q, the test dorsal root shock falls progressively later with respect to the pyramidal train. Two-neuron-arc discharges are identified by arrows.

In Fig. 10 P and Q are of interest because they show, in the absence of occlusion at the motoneuron, that higher order reflex discharges (conspicuously the three-neuron-arc discharge) are strongly facilitated after facilitation in the two-neuron-arc is no longer in evidence. The period during which facilitation at the internuncial level of the three-neuron-arc, including certainly the intermediate gray nucleus of Cajal, may be demonstrated, by the use of bisegmental testing reflex discharges, parallels the discharge period of the external basilar region. Undoubtedly the two events are related. Furthermore, Fig. 10 shows that the internuncial level of the three-neuron-arc receives facilitating

impulses for a longer period of time than it discharges impulses in turn to the motoneuron pool. Thus the evidence of Fig. 10 agrees closely with that of Fig. 8, employing similar conditions of stimulation in another preparation, and again points to the intermediate region (internuncial level of the three-neuron-arc) as the locus at which failure of the spinal mechanism to transmit pyramidal excitatory action first occurs.

The time interval between the onset of facilitation of the three-neuron arcs and of the two-neurons arcs is a measure of the time between the beginning of impulse arrival in the intermediate gray nucleus, and the beginning of impulse arrival in the motoneuron pool. Since the discharge of impulses in the intermediate region coincides with the arrival of impulses in the motoneuron pool, and since the two groups of neurons are synaptically related, the time difference between facilitation of three and two-neuron-arc discharges (Figs. 9 and 10) may be taken as a measure of the "nuclear delay"* in the intermediate nucleus.

Since the total latency for excitation at the motoneuron level is so long, it becomes of interest to determine if possible the shortest functional pathway that may be established by later volleys in a pyramidal tetanus, working on the background of activity supplied by antecedent volleys. An estimate may be reached by measuring the latency from a specific shock in the train of shocks to the onset in the motor nucleus of an effect attributable to that shock. For example, the onset of facilitation in Fig. 8, after 12 msec. total latency, is in part the result of the application of the third pyramidal shock 4.8 msec. after the initial shock of the series (Fig. 8, curve 3). This is the case since the first two pyramidal shocks had no tangible effect on the motoneurons (Fig. 8, curves 1 and 2). The resulting specific latency for the third shock amounts to approximately 7.2 msec. An accurate estimate of the specific latencies for the fourth, fifth, and sixth shocks in the experiment illustrated in Fig. 8 is not possible. Figure 11, therefore, presents curves from another experiment, which embody many more individual observations. As in Fig. 8, two pyramidal shocks had no measurable effect on the motor pool. The third shock of the train produced, by intensification and spread, a period of facilitation in the motor pool beginning at A. The horizontal displacement of arrow 3-A denotes the specific latency between the third shock and its effect in the motor

* Nuclear delay may be defined as the observed discrepancy in time between the arrival of impulses in a nucleus and the discharge of impulses from that nucleus. Thus it might be the equivalent of the recruitment period or even of a utilization period; it could not be the equivalent of synaptic delay (cf. Lorente de Nó, 1935). Nuclear delay is a function of the presynaptic elements of the nucleus because, when the intermediate nucleus is activated (i) through the pyramidal system, nuclear delay amounts to 3 or more msec.; but when activated (ii) through primary afferent collaterals, nuclear delay can occupy no more than a fraction of a msec. A unique and definitive account of the significance of nuclear delay as determined above is not apparent from the determinations themselves.

pool (approximately 7.3 msec.). On the graph are shown the facilitation curves resulting from 5 pyramidal shocks (circles) and from 6 pyramidal shocks (crosses). Since the two curves are superimposed, the point of divergence indicates the time at which the effect of the sixth pyramidal shock is first exerted on the motoneurons. Had the sixth shock maintained the same specific latency as that of the third, the facilitation curves of Fig. 11 would diverge at B, since the horizontal displacement of arrow 6-B was drawn to equal that of arrow 3-A. Such is obviously not the case. The specific latency

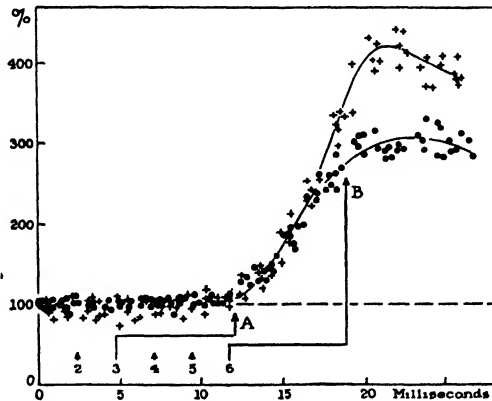


FIG. 11. Facilitation of motoneurons by 5 pyramidal shocks (circles) and 6 pyramidal shocks (crosses). The curves are constructed as in Fig. 8, the two-neuron-arc discharge again being used as a test. The arrows numbered successively at the bottom indicate the timing of the pyramidal shocks. The interval between the time of the sixth shock and the point of divergence of the two facilitation curves denotes the specific latency between the sixth shock and its effect in the motor pool. Arrow 3-A denotes the specific latency for the third pyramidal shock. Arrow 6-B has the same time span as Arrow 3-A. Note that the curves diverge before B; hence there is progressive shortening of latency in the spinal mechanism.

for the sixth shock is approximately 1.5 to 2 msec. shorter, *i.e.* 5.3 to 5.8 msec. in duration. Of this specific latency 4 to 4.5 msec. is attributable to pyramidal tract conduction, assuming the more rapidly conducting tract fibers to be involved. The remainder (0.8 to 1.8 msec.) is lost in the spinal mechanism, and it is of such time dimension as to suggest indirect pyramidal action on the motoneurons through internuncial relays. Since the present argument is based largely on time relationships, it will be seen that it is not necessary to assume the intercalation of interneurons between the more slowly conducting pyramidal fibers and the motoneurons. The action on motoneurons of direct pyramidal connections, if they exist, must be small, for it has not been detected by the use of single pyramidal volleys.

The discharge of motoneurons in response to pyramidal stimulation. In many

of the experiments of the present series a regular tonic discharge of impulses from the motoneurons has been detected readily by means of recording leads placed on a ventral root (Fig. 12A). Tonic innervation is well-known in the decerebrated preparation (decerebrate rigidity—Sherrington, 1897; cf. Denny-Brown, 1929; Adrian and Bronk, 1929). However, the tonic discharge of spinal motoneurons under the conditions of the present experiments is not a manifestation of decerebrate rigidity, for the vestibular and reticular pathways are severed (cf. Fulton, Liddell, and Rioch, 1930a, b), nor can it be due to maintained discharges from the cerebral cortex (Adrian and Moruzzi, 1939), because the brain stem is transected routinely at the colliculi. There remains the continuous flow of afferent impulses entering through the dorsal roots, previously mentioned. The tonic afferent discharge is probably the major factor in maintaining the tonic motoneuron discharge in the spinal animal. The tonic motoneuron discharge seen in these preparations is probably related to the flexor rigidity of Dusser de Barenne and Koskoff (1932, 1934). It may be noted that the spinal clamps that have been used in these experiments hold the preparation in a position appropriate for the development of flexor rigidity.

A prolonged* pyramidal tetanus (245 msec. duration) evokes after 20 to 30 msec. latency a discharge of motoneurons which takes the form of an increase in the level of the pre-existing tonic discharge (Fig. 12B). The added discharge often shows a maximum at 35 to 50 msec., following which is a depression and a secondary increase. After the cessation of the pyramidal stimulation, the duration of which is indicated by the horizontal line immediately below record 12C, the motoneurons "afterdischarge" for several hundred milliseconds.

It is difficult to estimate the part played by the pyramidal system *per se* in these prolonged phenomena, for once movement begins, locally engendered activity, reflecting from the periphery, may supervene to reinforce the pyramidally evoked activity. For instance, after record 12B was taken curare was administered to the preparation until movement was no longer observed. Figure 12C illustrates the discharge of motoneurons obtained by the pyramidal tetanus in the paralyzed preparation. The tonic activity is less, as is the activity evoked by the pyramidal stimulation. Note, however, that the motoneurons still maintain an afterdischarge for several hundred msec. in the curarized preparation. A comparison of Fig. 12B and C demonstrates in an elementary way the reinforcing action of secondary peripherally evoked activity.

Inhibition. Inhibitory actions frequently have been attributed to the

* These stimulations are not prolonged in the sense usually accepted in studies on the pyramidal system, nor are the frequencies employed throughout comparable with those usually employed. The reason for this of course is the purpose underlying the experiments, which likewise is different.

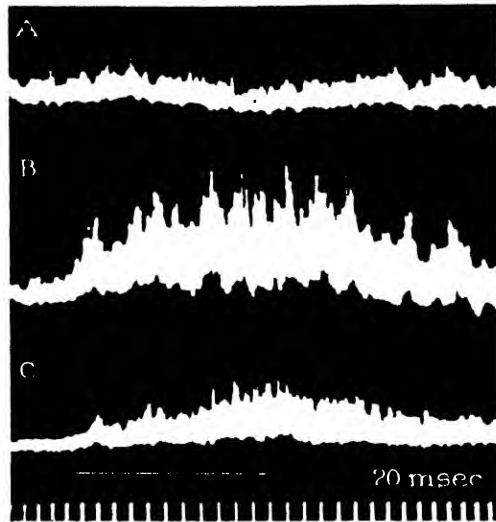


FIG. 12. Records from ventral root. A, tonic discharge of motoneurons (spinal preparation). B, motoneuron discharge resulting from pyramidal stimulation of 245 msec. duration. C, and B, but after prevention of movement by curare. Note afterdischarge in B and C. The horizontal line below C indicates the duration of the pyramidal stimulation in B and C. Further details in text.

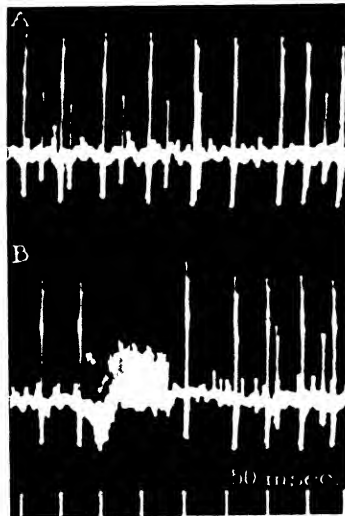


FIG. 13. Inhibition of tonic interneuron activity by pyramidal activity (100 msec. tetanic stimulation). Note the different times at which various elements return to activity.

pyramidal tract. Figure 13 presents one of several instances in which activity recorded from the intermediate gray substance was suspended rather than initiated or intensified by pyramidal stimulation. Record A of Fig. 13 illustrates the activity which was recorded in the absence of specific stimulation.* Record B of Fig. 13 shows the suspension of activity brought about by a pyramidal tetanus of approximately 100 msec. duration. Other units will be brought into activity during the time that the units at present under discussion are "inhibited" (cf. Figs. 5 to 7). Granting anatomical connection between the "inhibited" units and the motoneurons, the motoneuron discharges of Fig. 12 represent the final resultant of simultaneously occurring excitatory and inhibitory actions (direct or indirect, Lloyd, 1941b). It is not possible from the present experiments to determine whether or not the operation of reciprocal innervation, herein demonstrated for interneurons, is maintained within the motor pool by specific distribution of internuncial axons. The observations of Fig. 13 throw no light on the nature of the fundamental processes involved, which is in consequence not discussed.

Functional organization of the spinal mechanism of the pyramidal tract. Figure 14 presents in diagrammatic form a summary of functional connections between fibers of the pyramidal system, primary afferent collaterals, and neurons of the spinal cord, as they appear in the light of the foregoing observations. The diagram is admittedly incomplete and imperfect. The pyramidal fibers (P) are pictured as ending most prominently on the cells of the external basilar region (E). Possible connections to the solitary cells (S) and some cells of the intermediate region are indicated. There is little indication from the present experiments that pyramidal fibers end on motoneurons (M.N.). In consequence no connections are included in Fig. 14. The cells of the external basilar region (E) relay activity to the solitary cells (S) and to the intermediate region (I and I₂). The intermediate gray nucleus of Cajal is represented (I). Primary afferent collaterals (P.A.) convey activity through branches 3 and 3a to interneurons, which in turn supply motoneurons (M.N.) thus completing three-neuron reflex arcs. Furthermore, primary afferent collaterals (P.A.) through branch 2 to the motoneurons (M.N.) complete the two-neuron reflex arcs.

Emphasis must be placed on the fact that activity within the spinal gray substance is continuous from the arrival of the first tract impulses as the result of pyramidal stimulation until the spinal mechanism reaches the highest

* One is inclined to accept the physiological nature of the internuncial discharges "spontaneously" occurring in Fig. 13, for they may continue unabated for hours with amplitude and phase relationships of the spike potentials preserved. Likewise, since the preparations exhibit a constant afferent inflow and a tonic motoneuron discharge, there is no reason to suppose that the interneurons would not display activity if the recording microelectrode were not in position.

activity level possible under the conditions of the experiment. Within the stroma of continuous activity certain neurons, or groups of neurons, become

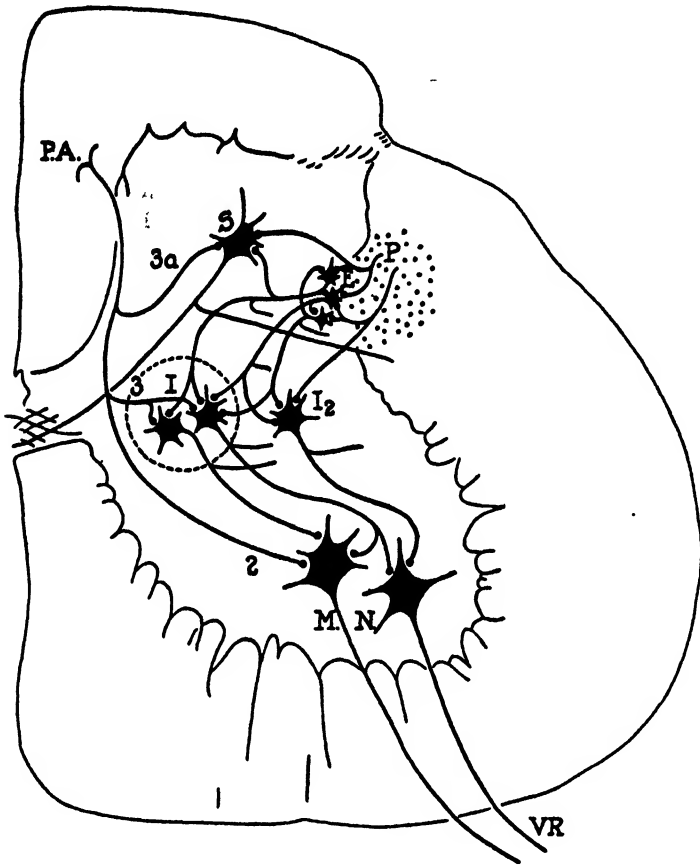


FIG. 14. Functional organization of the spinal mechanism of the pyramidal tract. Connections from the pyramidal tract and primary afferent collaterals are represented. E, small cells of the external basilar region; I, intermediate gray nucleus of Cajal; I₂, other neurons of the intermediate region; M.N., motoneurons; P, pyramidal tract and fibers; P.A., primary afferent collaterals; S, solitary cells of the dorsal horn; V.R., ventral root; 2, 3, and 3A, terminal collaterals of the primary afferent system.

active at well defined and reproducible time intervals after the onset of the pyramidal stimulation. The dynamic characteristics of the spinal mechanism of the pyramidal tract may be summarized by a brief recapitulation of two type experiments. In the first type experiment a single pyramidal volley is used. Pyramidal impulses first enter the lumbo-sacral cord at 4.5 msec. after the pyramidal shock and continue to do so for possibly 10 or more milliseconds

(Fig. 2). They are accompanied by a burst of nuclear activity in the external basilar region (Fig. 3). Other types of internuncial activity do not ensue, nor is reflex activity affected demonstrably throughout the activity period. Presumably following a single pyramidal shock, the impulses of the external basilar region (E in Fig. 14) circulate within the nucleus itself, extranuclear discharges through longer collaterals to other nuclei being negligible. In the second type experiment several pyramidal shocks are used. The minimal latency of pyramidal conduction is 4.5 msec. as before, but tract activity and external basilar region activity are intensified by the added pyramidal shocks. The contribution of impulses to other internuncial nuclei becomes sufficiently great to result in measurable facilitation at the internuncial level of the three-neuron reflex arc at 9 msec. after the onset of the pyramidal stimulation. Thus approximately 4.5 msec. are lost in the dorsal horn. An additional 3 msec. are lost in nuclear delay at the intermediate region before intermediate neurons discharge and motoneurons are facilitated. Thus the total latent period for facilitation of motoneurons (12 msec.) is accounted for by (i), tract conduction (4.5 msec.); (ii), dorsal horn latency (4.5 msec.); and (iii), nuclear delay at the intermediate level (3 msec.).

As activity spreads slowly through the spinal gray substance, progressively shorter functional pathways are opened to the pyramidal impulses, so that the time lost in the internuncial pools of the spinal gray substance is reduced from approximately 7.5 msec. to approximately 1.0 msec. (Fig. 11).

When pyramidally evoked activity reaches the motoneurons, the motoneuron discharge realized through three-neuron reflex arcs may be facilitated at both the interneuron and motoneuron levels. If a large two-neuron-arc response is present in the test reflex discharge, occlusion of the three-neuron-arc discharge at the motoneuron level counteracts the facilitatory action of the pyramidal stimulation.

SUMMARY

A method is described whereby a controlled pyramidal volley may be delivered into the spinal cord. Using this method, an attempt is made to outline the functional organization of the spinal mechanism under the conditions of pyramidal stimulation. A summary of this organization is presented in connection with Fig. 14. Cats were used.

Pyramidal activity is distributed along the length of the spinal cord by the pyramidal tract fibers. The most rapidly conducting of these have a velocity between 60 and 65 M per sec. The lower limit of pyramidal fiber velocities is uncertain, but dispersion of a volley, initially synchronous at the medullary level, is sufficient to produce a discharge of pyramidal impulses into the lumbar cord lasting many milliseconds.

Interneurons of the spinal cord are of paramount importance in effecting

the distribution of pyramidal activity. Small nuclear elements, in close proximity with the tract, appear to constitute the initial internuncial relays. The final or premotoneuron internuncial relays lie within the intermediate region. Facilitation of motoneurons, as tested through primary afferent volleys, parallels activity in the intermediate region. In view of known anatomical connections a causal relationship between intermediate internuncial discharges and excitation of motoneurons appears to be established.

A nuclear delay of several milliseconds exists at the intermediate nucleus under the conditions of pyramidal excitation. The same nucleus activated through primary afferent collaterals has a nuclear delay of but a fraction of a millisecond. The differences are a function of the presynaptic elements rather than of the postsynaptic elements.

As pyramidal activity is intensified, shortening of the functional paths interposed between pyramidal fibers and motoneurons occurs. The rate of shortening is related to the frequency of pyramidal stimulation.

Whereas some interneurons of the intermediate region are activated by pyramidal stimulation, others are inhibited (reciprocal innervation).

Tonic discharge of spinal motoneurons occurs in the spinal state. This discharge is probably related to the flexor rigidity of Dusser de Barenne and Koskoff. The constant arrival of impulses from the periphery must be considered as an important factor in the maintenance of the tonic discharge in the spinal preparation.

Motoneuron discharge resulting from pyramidal activity is highly asynchronous, although without doubt some motoneuron units carry the pyramidal stimulus frequency.

Spinal reflex reinforcement of pyramidal activity begins with the onset of movement, and it may be important in determining the character of motor performance arising from cortical stimulations.

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STIMULATION OF PERIPHERAL NERVE TERMINATIONS BY ACTIVE MUSCLE

By DAVID P. C. LLOYD

(From the Laboratories of The Rockefeller Institute for Medical Research)

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A single shock applied to the distal segment of a severed ventral root results in the appearance of a group of impulses which reflect from the periphery into a fraction of the stimulated ventral root fibers, into unstimulated but neighboring ventral root fibers, and into fibers of the corresponding dorsal roots (9). Centripetal discharges have been observed in ventral roots or motor nerves of eserinated preparations (10, 4, 6, 5). These centripetal discharges have been regarded as arising at the motor nerve endings (under the influence of eserine) and coursing both orthodromically into the muscle there to cause the repetitive responses characteristic of eserinated muscle (3) and antidromically along the motor nerve (ventral root) where they may be recorded. Two lines of evidence stand contrary to this view. Eccles, Katz and Kuffler, (5) point out that the association of "repetitive" motor nerve responses with repetitive muscle response is not invariable. Secondly the centripetal responses have been observed in non-eserinated preparations, and in root fibers other than those occupied by the efferent volley to the muscles.

Afferent activity in dorsal roots would naturally ensue upon active contraction of the muscles (12), but the dorsal root activity at present under consideration does not possess the features to be expected in the case of discharges from any known tension receptors.

It is the purpose of the present study to show the origin and nature of the secondary centripetal discharges in the normal (*i.e.* non-eserinated) preparation. Some of the pertinent observations have been presented briefly in a preliminary note (9).

The animals used in these experiments were cats, and two rabbits, one of which showed characteristic secondary centripetal activity. According to Feng and Li (6) normal "after discharge" occurs also in rats. The animals were narcotized with Dial (0.5 ml. per kg.). Selected dorsal and ventral roots were exposed by laminectomy and severed from their connection with the spinal cord for the purpose of placing stimulating and recording electrodes.

When the seventh lumbar ventral root (L.7 V.R.) is stimulated by a single maximal shock, recording leads on that root reveal the spike potential of the stimulated volley (the primary motor volley) followed, after some 2.5 msec., by an asynchronous discharge lasting, on the average, ca. 5 msec. (Fig. 1A). An essentially similar result is obtained if the first sacral ventral

root (S.1 V.R.) is substituted (Fig. 1C). The records of Fig. 1 were all obtained with one of the recording leads at the killed end of the root in question. When both recording leads are placed on the intact root distal to the stimulating electrodes the phases of the potentials of the primary motor volley and of the secondary discharges are reversed indicating clearly that the primary motor volley passes distally to the muscles, whereas the secondary impulses are centripetal (cf. Lloyd, 9, Fig. 1F, L, M).

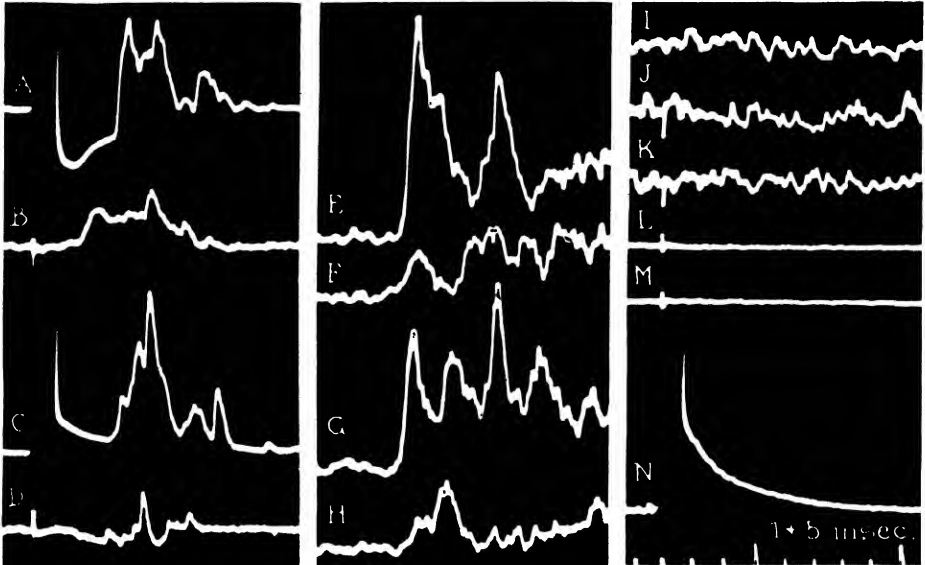


FIG. 1. Distribution of secondary centripetal discharges in ventral and dorsal roots following stimulation of ventral roots. Time in 1 and 5 msec. divisions below record N. In all figures where there are two time designations, these are for the small and large divisions respectively. Further description in text.

The discharge of 1B of Fig. 1 is recorded from the S.1 V.R., and results from a single shock delivered to the L.7 V.R. The primary motor volley is of course not recorded. The secondary centripetal impulses are present, although the discharge is not as intense as it is in the L.7 V.R. The latency of the secondary centripetal discharge is shorter in 1B than in 1A or 1C, being ca. 1.6 msec., as compared with 2.5 msec. The differential is not always so great, although it is generally present unless other factors are operative. Figure 1D shows the secondary centripetal discharge in the L.7 V.R. resulting from a S.1 V.R. shock.

From a consideration of Fig. 1 (A to D) it becomes apparent that the occurrence of secondary centripetal discharges in ventral roots does not de-

pend upon the identity of the nerve structures transmitting the primary motor volley and the secondary centripetal discharges. Secondary centripetal discharges, on the other hand, are not encountered if several segments intervene between the "donor" and "recipient" ventral roots. The spatial limits to the distribution of the secondary centripetal discharges appear, therefore, to be set by the extent of spatial fusion in the distribution of the several ventral roots to the muscles. Spatial fusion is extensive in the limb musculature (16), which no doubt accounts for the substantial secondary centripetal discharges encountered in ventral roots adjacent to a root occupied by the primary motor volley. Inasmuch as the secondary centripetal discharges do occur in roots not occupied by the primary motor volley, they are not repetitive discharges with respect to that volley.

Secondary centripetal impulses occur in corresponding dorsal roots with comparable latency. Figure 1(E, F) shows these impulses recorded from the seventh lumbar dorsal root (L.7 D.R.) and the first sacral dorsal root (S.1 D.R.) following single shock stimulation of the L.7 V.R. Similarly Fig. 1(G), H shows secondary centripetal responses in the S.1 D.R. and L.7 D.R. to stimulation of the S.1 V.R. The earliest dorsal root secondary centripetal responses occur usually after 1.6-1.7 msec. latency. Again, as in the case of the ventral root secondary centripetal responses, it will be noted that the dorsal root secondary centripetal responses are greatest when the ventral root of the same segment is stimulated.

Figure 1 (J to N) illustrates the ineffectiveness of centrifugal volleys in dorsal roots with respect to the initiation of secondary centripetal discharges. In 1J, the L.7 D.R. is stimulated while recording from the S.1 D.R. In 1K, stimulating and recording leads are reversed. The activity recorded in J and K is similar to that recorded in I without stimulation, and represents the normal and constant flow of afferent activity over the dorsal roots (1). For records L and M recording leads were placed on the L.7 V.R. and S.1 V.R. respectively, in each case the dorsal root of the same segment being stimulated. Again no secondary centripetal activity results. Finally in N, both stimulating and recording electrodes were placed on the S.1 D.R. A primary spike potential is recorded unaccompanied, however, by any sign of secondary centripetal activity.

It is clear from the observations of Fig. 1 that secondary centripetal discharges result only when a ventral root is stimulated and occur in fibers, both dorsal and ventral root, having a degree of anatomical fusion of peripheral distribution.

It has been noted that the latency for secondary centripetal discharge in a stimulated ventral root is longer than that in unstimulated ventral roots or in dorsal roots. Differences other than latency exist between the secondary centripetal discharges of stimulated and unstimulated roots. These are: (i)

the relative size of those discharges, and (ii) the size of the primary motor volley necessary to procure threshold secondary centripetal discharges in dorsal and stimulated ventral root fibers.

Figure 2 presents a graph relating the amplitudes of the dorsal root and stimulated ventral root secondary centripetal discharges, plotted in similar arbitrary units but on different scales, to the size of the primary ventral root volley expressed in per cent of maximum. The dorsal root secondary centripetal discharge is much greater than that of the ventral root. This fact may be appreciated also in Fig. 1, 3 and 4 by noting that the ventral root responses have been recorded regularly at an amplification five times that

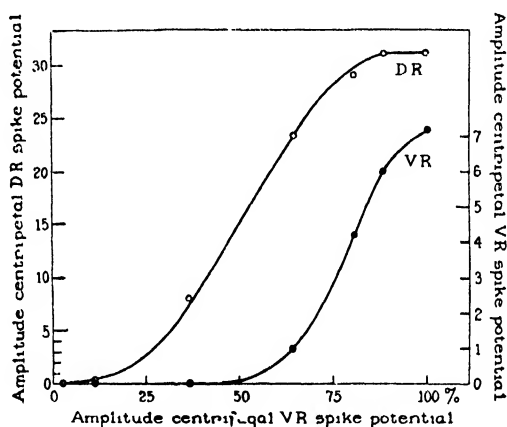


FIG. 2. Graph relating the size and threshold of secondary centripetal discharges in a dorsal root and a stimulated ventral root. The first spike potential of the discharges was selected for measurement and illustration merely because some part of the complex discharge must be chosen. Other parts of the secondary centripetal discharges respond similarly to ventral root shocks of increasing intensity.

used for the dorsal root responses. Figure 3 further indicates the much lower threshold for production of the dorsal root secondary centripetal responses than for the production of the ventral root secondary centripetal responses.

Thus there are differences in latency, size and threshold to distinguish between the secondary centripetal discharges in dorsal and stimulated ventral roots. These differences are consentient in indicating that the motor fibers, as a result of the passage of the primary motor volley, are partially refractory to the excitatory process causing the secondary centripetal discharges.

Elements of the secondary centripetal discharge. It has been shown that the secondary centripetal discharge is asynchronous, having a duration of ca. 5 msec. This could be due to repetitiveness, or to a variety of conduction times occasioned by circuits of various lengths. To decide between these two al-

ternatives, advantage is taken of the fact that the constituents of the lumbosacral cord break up into three major groups; the gluteal and other nerves innervating muscles about the hip, the hamstring group to muscles of the thigh, and the sciatic group to muscles of the leg. These groups are characterized by different average conduction distances between the spinal roots and the muscles concerned; each group may be interrupted in isolation from the others.

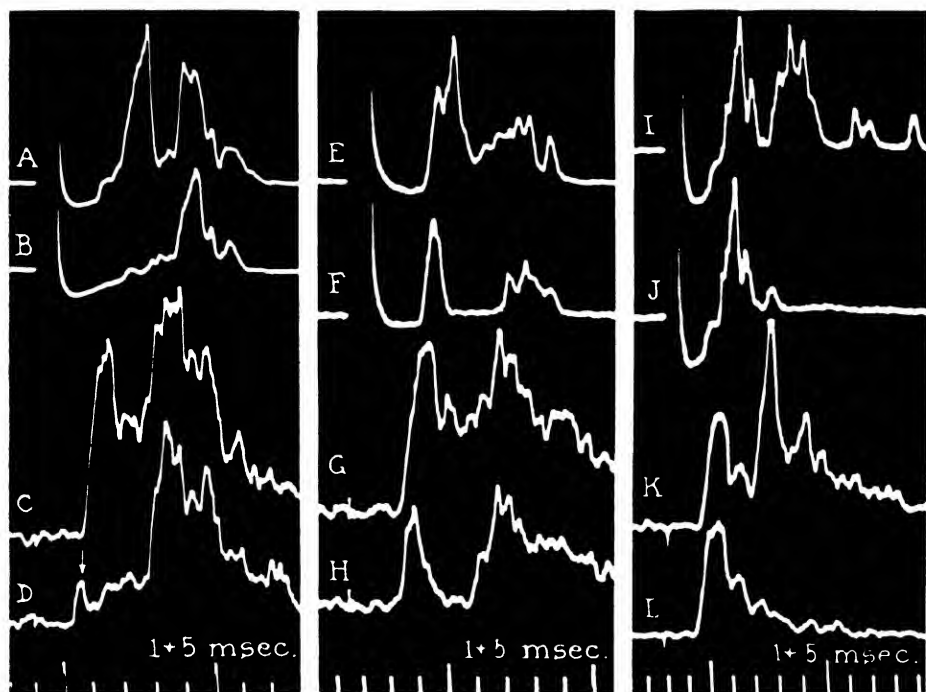


FIG. 3. Removal of specific segments of dorsal and ventral root secondary centripetal discharges by selective denervation of muscle groups. The spike potential introduced in record D after nerve section is a "denervation artifact."

The results of experiments in which selective denervation of these muscle groups was practised are illustrated in Fig. 3.

Figure 3 (A, E, I) shows the ventral root (L.7) secondary centripetal discharges in three experiments preceding surgical intervention in the leg. Similarly Fig. 3 (C, G, K) shows the dorsal root (also L.7) secondary centripetal discharges in the same three experiments and under the same conditions. Denervation of hip muscles changed the ventral root secondary centripetal discharge from that recorded in 3A to that recorded in 3B, and the dorsal root secondary centripetal discharge from that in 3C to that in 3D. The

initial segment of the secondary centripetal discharge is depleted in each case;* the terminal segment is retained.

When the hamstring nerves are severed the secondary centripetal discharge in ventral roots is changed from that recorded in 3E to that recorded in 3F, while the dorsal root secondary centripetal discharge is changed from that in 3G to that in 3H. Figure 3 (E to H) shows that the intermediate segment of the secondary centripetal discharge is obliterated by denervation of the hamstring group. In similar fashion the observations 3I to 3L show that the terminal portion of the secondary centripetal discharge is removed by section of the sciatic nerve (compare J with I and L with K), whereas the initial portion is retained almost perfectly intact.

There can be little doubt that the significant factor for dispersion of the secondary centripetal discharge is variety of conduction distance to and from the several groups of nerve endings, efferent and afferent, and that the latency of each segment of the discharge is related to the total conduction distance involved. It will be noted that the major discharges of any particular group remaining intact occur with a shorter latency in the dorsal roots than in the stimulated ventral roots. The initiation of the later segments of the secondary centripetal discharge in the stimulated ventral root therefore appears to be subject to the same conditions of partial refractoriness that hold for the initiation of the shortest latency secondary centripetal discharges (cf. Fig. 1 and 2). The latency differential between dorsal and stimulated ventral root secondary centripetal discharges would necessarily hold for each segment of those discharges if the secondary centripetal discharges are initiated in the terminal regions of "recipient" fibers at a relatively fixed time with respect to the arrival of the primary motor volley at the various motor fiber terminations, conduction distance being the major variable.

In occasional experiments one or two centripetal discharges occur in a few fibers after the completion of the major secondary centripetal discharge (cf. for example Fig. 3I). It is possible that these atypical discharges are repetitive in nature.

Factors contributing to the latency of secondary centripetal discharges. Nerve conduction time is a significant factor in latency of secondary centripetal discharges, but nerve conduction alone cannot account in full for the latency

* The spike potential indicated by an arrow in Fig. 3D serves to illustrate one of the sources of confusion inherent in denervation experiments. This spike potential is not present in the normal record 3C. It has appeared *de novo* as a result of nerve section. Its latency is ca. 0.5 msec. shorter than that of the first activity in 3C. It may be identified as due to stimulation of D. R. fibers at the cut end of the common nerve trunk by closely intermingled active V. R. fibers (cf. 7). Another source of error arises in the stimulation of cut nerve ends by active muscle (11). Careful preparation usually reduces these "artefacts" to insignificance.

of even the earliest of these discharges for, as seen in column 7 of Table 1, the latency of secondary centripetal discharges is 0.54 to 0.8 msec. longer than that of "Hering phenomena" (stimulation of nerve by nerve) conducted over nerve paths of comparable length. The discrepancy between the latency of secondary centripetal discharge and the Hering phenomenon is also seen in Fig. 3 (C, D). The exclusion of nerve stimulation by nerve as an explanation of the secondary centripetal discharges does not rest upon the evidence of differential latency alone. The fact that secondary centripetal discharges reflect into a *maximally* stimulated "donor" ventral root (Fig. 1, 3 and 4), whereas Hering phenomena do not is a powerful argument against an analogy between the two phenomena. Accordingly the stimulating agent causing the secondary centripetal discharges and that causing the Hering

TABLE 1

Nerve-muscle group	Time from stimulus to muscle action potential	Conduction time, muscle to root	Sum of 2 and 3	Latency of secondary centripetal discharge	Latency of Hering phenomenon on sciatic N. at equivalent conduction distances	Excess over 6
1	2	3	4	5	6	7
Hip*	1.1 1.15	0.5 0.45	1.6 1.6	1.64 1.66†	1.1 (10 cm.)	0.54-0.56
Hamstring	1.5	0.76	2.26	2.3	1.76 (16 cm.)	0.54
Sciatic	2.0	1.4	3.4	3.5‡	2.7 (24 cm.)	0.8

* This experiment is illustrated in the preliminary paper (Lloyd, 9, Fig. 1B, C, D, E).

† This value was obtained at the beginning of the experiment. When the active muscle field was restricted by nerve section a denervation artefact appeared with shorter latency, obscuring the normal onset of the secondary centripetal discharge.

‡ The records from which this figure was obtained contain a persistent denervation artefact emanating from the hip region. This artefact, however, did not encroach upon the secondary centripetal discharge.

phenomenon may not be homologized for the former outlasts the absolutely refractory period of the nerve fibers carrying the primary motor volley with an intensity sufficient to reexcite those same fibers during partial refractoriness. In the case of the Hering phenomenon the stimulating agent is coincident with the arrival of the donor nerve volley (13) and does not persist long enough to clear refractoriness of the donor nerve fibers. The observations presented in Table 1 indicate that it is the time dissipated in neuromuscular delay before the stimulating agent causing the secondary centripetal discharges begins to act that allows the agent to clear the refractoriness of the nerve fibers occupied by the primary motor volley.

Table 1 shows the results of experiments designed to account for the duration of the latent period of the secondary centripetal discharges and to locate the time at which these discharges are initiated at the periphery in

relation to the time sequence of events following upon a ventral root shock. A comparison of the figures in columns 4 and 5 indicates that the latency of a selected segment of the secondary centripetal discharge is approximately equal to the sum of conduction time to the muscle, neuromuscular delay and conduction from the muscle to the root recording leads. The residual time (0.04–0.1 msec.) may be accounted for as utilization time. The earliest secondary centripetal discharges are initiated, therefore, shortly after the onset of the muscle action potential at the junctional region.

Although the total secondary centripetal discharge in the intact leg lasts for ca. 5 msec., the discharge from a restricted muscular field is much shorter

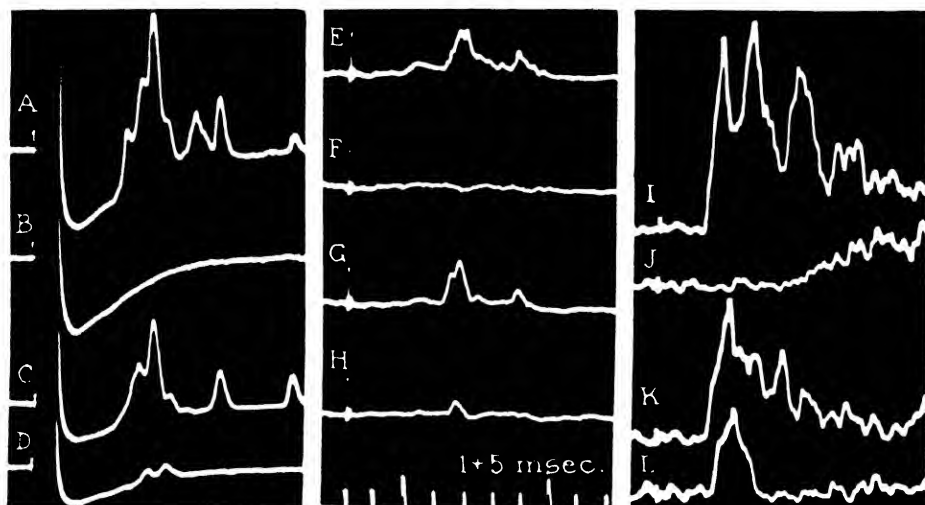


FIG. 4. Removal of secondary centripetal discharges by curare. Inhibition of secondary centripetal discharges by the Wedensky effect at the neuromuscular junction of partially curarized muscle.

(Fig. 3). Restriction of the donor muscular field by denervation (Fig. 3 and Lloyd, 9, Fig. 1B) or by the use of threshold responses (for example in Fig. 6) shows that the unit secondary centripetal discharge has a total duration of 1 to 1.3 msec. at the recording leads. Allowing for the duration of the individual nerve spikes in the secondary centripetal discharge, but not for conduction dispersion, it appears that the last secondary centripetal discharges of a homogeneous group are initiated in the nerve endings within ca. 0.5 to 0.8 msec. of the onset of the first of such discharges. This time coincides approximately with the crest time of the muscle action potential. The secondary centripetal discharges are initiated, therefore, by processes approximately equal in duration to and contemporaneous with the ascending phase of the muscle action potential at or near the junctional region.

Contributory evidence for the participation of neuromuscular transmission

and muscle action in the sequence of events leading to secondary centripetal discharges. If muscle action is a step in the production of secondary centripetal discharges then these discharges should be susceptible to modification in a predictable manner by agents and procedures known to influence neuromuscular transmission and muscle action.

(1) *The action of curare.* Curare removes the secondary centripetal discharges even when administered in doses which are not quite sufficient for the complete blocking of muscular response. Figure 4 (A, E, I) shows the secondary centripetal discharges in L.7 V.R., S.1 V.R. and L.7 D.R. in response to a L.7 V.R. shock. Figure 4 (B, F, J) recorded after the administration of curare, illustrates the removal of the secondary centripetal discharges in each case. Figure 4J is of interest for it reveals a response of recruiting nature and of much longer latency still present after a degree of curarization sufficient to remove the secondary centripetal discharges in the dorsal root and elsewhere. Positive identification is not easy, but it is most likely that this response represents afferent activity initiated by the residual muscle contraction acting on tension receptors.

One might suppose that the secondary centripetal discharges should disappear only with the advent of complete curarization. Reference to Fig. 2, however, shows that the first (and lowest threshold) secondary centripetal responses appear as a result of primary motor volleys between 12 and 35 per cent of maximum size. Asynchronous recruiting afferent activity, comparable with that in Fig. 4J, appears following near threshold motor volleys. Differential threshold then is adequate to account for the segregation by curare of later afferent activity from the secondary centripetal discharge.

With small doses of curare the secondary centripetal activity is present, with latency unaltered, but with intensity diminished (compare Fig. 4C, G, K with the control observations 4A, E, I). If two shocks are delivered in succession to a ventral root of a partially curarized preparation at intervals up to 5 or more sec., the secondary centripetal activity elicited by the second of such pairs of shocks is markedly reduced. The pairs of responses C and D, G and H, and K and L were recorded at an interval of 1 sec. in each case. The reduction in the secondary centripetal activity elicited by the second shock of each pair is readily apparent. The effect is clearly a reflection of the prolonged "Wedensky type" inhibition characteristic of partially curarized muscle (8, 2, 15).

(2) *The action of eserine.* It has been known for some time that the response of eserinated muscle to a single motor volley is repetitive (3). Likewise it has been in the eserinated preparation that repetitive centripetal activity has been observed (10, 6, 5). It remains to be shown, however, that the repetitive nerve activity is primarily grafted onto the secondary centripetal activity present before the administration of eserine. Figure 5 illustrates the secondary centripetal discharges evoked in one half of the L.7

V.R. by stimulation of the other half before (A) and after (B and C) the administration of eserine (0.5 mg. intravenous). Comparison of 5A and 5B reveals that the original secondary centripetal discharge group is retained and that the repetitive discharge is added thereto. Figure 5C, similar to 5B but recorded on a slower time axis, illustrates how the repetitive discharge is prolonged in time.

It is difficult in view of the other evidence presented here to escape the conclusion that the repetitive centripetal discharges recorded under the influence of eserine result from the repetitive response of the muscles to a single nerve volley, rather than *vice versa* as proposed by Masland and Wigton. The present interpretation has the additional merit of accommodating the observation of Eccles, Katz and Kuffler (5) that repetitive muscle responses caused by eserine frequently occur without discharges into the motor nerve. The explanation of the variable association would lie in the intensity of the muscle action necessary to secure centripetal discharges (cf. discussion in connection with Fig. 2 and 4).

The occurrence of centripetal discharges associated with the spontaneous fasciculation of eserinated cat's muscle, first observed by Masland and Wigton, has been confirmed.

(3) *The stages of neuromuscular transmission.* One of the prominent characteristics of neuromuscular transmission is the series of variations in muscle response to maintained tetanic stimulation of the motor nerve. These variations have recently been systematized by Rosenblueth and coworkers (14) into five stages of neuromuscular transmission. The influence of these stages of neuromuscular transmission on the initiation of secondary centripetal discharges has been observed, in varying degree, in all the preparations made for that purpose. The second stage is often absent, but the stages representing "treppe" and "fatigue" are regularly present. The optimal preparation for observing the treppe phases (stages 1 and 3) is that employing separate ventral roots for stimulating and recording.

Observations D to K of Fig. 5 illustrate the influence of the initial stages of neuromuscular transmission on secondary centripetal discharges and were recorded in the following manner. A single response (5D) was recorded. The succeeding responses were recorded at intervals as a standing wave, the stimulus being synchronized with the sweep, the stimulus frequency approximating 50 per sec. There are slight variations in individual responses in addition to the slower trends; hence the double exposure effect in records E to K. In the experiment illustrated the second stage is present resulting in an initial decrease in the secondary centripetal discharge, succeeded by an increase (third stage) and "fatigue" (fourth stage). The experiment was not prolonged to the point of observing the fifth stage.

In addition to the slow changes in intensity of secondary centripetal activity, Fig. 5 illustrates a fact which holds equally for secondary cen-

tripetal discharges in dorsal roots, stimulated and unstimulated ventral roots. The secondary centripetal discharges follow exactly the stimulus frequency at frequencies yielding mechanical fusion of muscle contraction. Granting that some aspect of muscle action is responsible for the initiation of secondary centripetal discharges, the last fact would tend to exclude muscle contraction as the stimulating agent. It is worth noting, since stimulation by muscle action currents is implied as the mechanism of secondary centripetal discharge in an earlier section, that the muscle action potentials remain distinct during a tetanus and furthermore that they undergo characteristic amplitude variations during the several stages of neuromuscular transmission (14).

The locus of stimulation of recipient nerve fibers. The observations on latency (Fig. 3 and Table 1) indicate that the recipient nerve fibers are stimu-

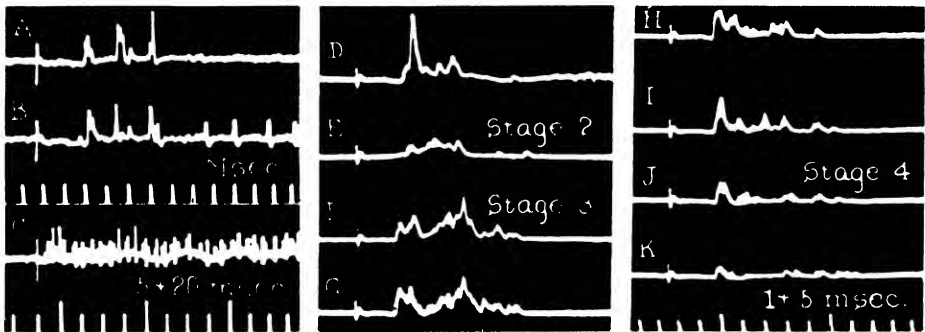


FIG. 5. Influence of eserine on secondary centripetal discharges (A, B, C). D to K - the secondary centripetal discharges reflect the stages of neuromuscular transmission. The time lines refer to the record or records immediately preceding.

lated at or near their terminations and not at random along some part of their course. In order to substantiate this view, control experiments to demonstrate that active muscles do not stimulate the *intact* nerve trunks (*i.e.* the preterminal portions of the nerve fibers) were fashioned as follows. In addition to the usual root preparation, a small window was made over the hamstring nerves which were located and severed. Otherwise the normal anatomical relationships within the leg were preserved. On stimulating a ventral root, centripetal discharges comparable with those illustrated in Fig. 3F resulted. Stimulating electrodes were applied to the distal segment of the severed hamstring nerves. On stimulation of the hamstring nerves, resulting in contraction of the hamstring muscles, no secondary centripetal discharge was recorded at the spinal roots although a great length of the sciatic nerve lay immediately subjacent to the active hamstring muscles.

Furthermore, stimulation of the hamstring nerves did not influence the secondary centripetal discharge resulting from stimulation at the spinal root.

Summation of two excitations by muscle. Figure 6 presents the results of an experiment designed to measure the summation interval of two excitations of nerve at the periphery by muscle action. The L.7 V.R. was split longitudinally so that together with the S.1 V.R. three portions of ventral root were available. Two of these were equipped with stimulating electrodes, through each pair of which a single shock could be delivered. In this way two

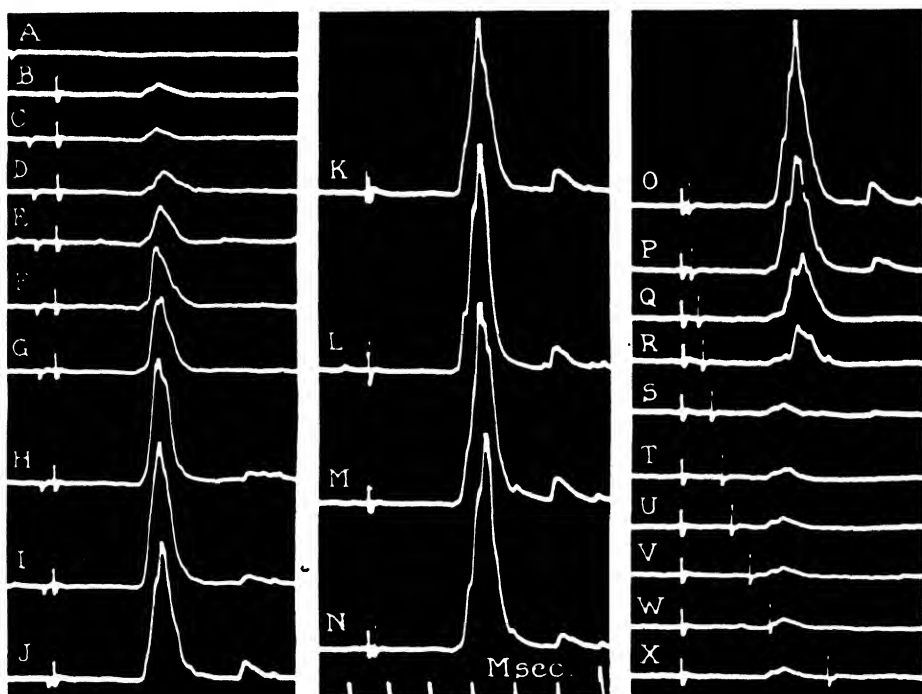


FIG. 6. The summation of peripheral excitations causing the secondary centripetal discharges.

groups of muscle fibers could be activated independently. The third portion of ventral root was provided with recording leads. By this device the recipient ventral root fibers were entirely free of primary motor volleys.

Records A and B of Fig. 6 show the results of the two ventral root shocks in isolation. The ventral root volley A is too small to elicit a secondary centripetal discharge in the recipient root, while the volley B alone evokes a small response. When the volleys A and B are synchronized (record 6L) a large response results, showing that volley A has a subliminal excitatory action on the recipient root endings. In records 6C to 6X the two shocks

have been delivered at intervals varying from A 0.7 msec. before B, to B 3.6 msec. before A. Summation of the first discharge group begins in 6D (A—0.6 msec.—B) and ends in 6S (B—0.8 msec.—A). The total summation period for this group then is ca. 1.4 msec.

A second group, arising distal to the first group, first appears in 6H and disappears in 6Q revealing a summation period of 0.9 msec. The second discharge group is absent, in each case, following the shocks in isolation. The full duration of the summation period cannot be determined from the appearance and disappearance of the second group, therefore, since the summated excitation could well be subliminal at each end of the period.

The summation period for excitations of nerve by muscle again suggests the brevity of the causal excitation process.

SUMMARY

When a muscle is activated indirectly by a motor nerve (ventral root) volley, centripetal volleys ensue. These may be recorded in the stimulated ventral root and in neighboring ventral and dorsal roots.

The latency of the secondary centripetal discharges is equal to the sum of conduction time from the stimulating electrodes to the muscle, neuromuscular delay, a short utilization period, and conduction time from the muscle to the recording leads. In the case of secondary centripetal discharges into a stimulated ventral root, the consequences of refractoriness add to the total latency.

The secondary centripetal discharges reflect faithfully the intervention of neuromuscular transmission and muscle action as steps in their causal sequence.

The secondary centripetal discharges are initiated by muscular processes approximately equal in duration to and coincident with the ascending phase of the muscle action potential at or near the junctional region.

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DESTRUCTION OF THIAMINE BY A SUBSTANCE IN CERTAIN FISH

By D. W. WOOLLEY

(From the Laboratories of The Rockefeller Institute for Medical Research)

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Green, Carlson, and Evans¹ recently have shown that foxes developed a severe disease when fed a diet containing raw carp and that this disease could be prevented by the feeding of thiamine. Since studies of the egg white injury factor and its reaction with biotin² were in progress in this laboratory at the time that the paper of Green *et al.* appeared,³ the possibility was considered that an "antithiamine" existed whose relation to thiamine was similar to that of the antibiotin factor to biotin. An attempt was therefore made to demonstrate an antithiamine in carp by chemical and biological procedures.

A freshly killed carp was ground and suspended in water, and 4 volumes of ethanol were added. The alcohol extract was found to contain no thiamine when examined by the method of Emmett *et al.*⁴ Furthermore, thiamine added to the fish could not be recovered. 100 gm. of various carps caused the disappearance of 150 to 190 γ of the vitamin when 200 γ were added. A suspension of carp which had been heated to the boiling point and cooled removed only half as much thiamine. Heating for 15 minutes at 15 pounds pressure destroyed all activity. Similar behavior was observed with solutions of the factor obtained as described below. Aqueous extracts were only one-fourth as active as the suspension of carp, while aqueous extracts of dialyzed suspensions were inactive toward thiamine. However, the active ingredient was not dialyzable. It could be extracted from the insoluble portion of the fish with 10 per cent NaCl.

¹ Green, R. G., Carlson, W. E., and Evans, C. A., *J. Nutrition*, **21**, 243 (1941).

² Eakin, R. E., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **140**, 535 (1941).

³ Woolley, D. W., and Longworth, L. G., *J. Biol. Chem.*, in press.

⁴ Emmett, A. D., Peacock, G., and Brown, R. A., *J. Biol. Chem.*, **135**, 131 (1940).

One-fourth of the activity of a fish was in the head, three-eighths in the viscera, and three-eighths in the torso.

An independent method of analysis was sought in order to establish these observations more securely and to extend them. After the examination of the responses of a number of micro-organisms it was found that the yeast *Endomyces vernalis* was suited to the purpose. When this organism was inoculated into the medium described by Snell *et al.*,⁵ from which thiamine was omitted, slight growth occurred. When thiamine was added, growth was as good as in a malt extract medium.⁶ By the use of a quantitative method based on these observations it was found that the aqueous extract of carp contained only 3 γ of thiamine and its pyrimidine and thiazole halves per 100 gm. of fish and that suspensions or NaCl extracts of carp destroyed thiamine.

The mode of destruction has not been established. Since the pyrimidine and thiazole halves together were as active as thiamine for *Endomyces* and since thiamine activity for this organism was destroyed by the preparation, the mode of action cannot be to split the molecule into these halves. The destruction was not instantaneous. A certain preparation destroyed no thiamine in zero time, 1.3 γ in 6 hours, 4.0 γ in 16 hours, 4.3 γ in 24 hours, at 25° (*Endomyces* method). Whether the action is an enzymic degradation or a slow formation of a thiamine-antithiamine complex has not yet been established.

1 mg. of the most active preparation thus far obtained by methods suggested above destroyed 1.8 γ of thiamine in 2 hours at 25°.

⁵ Snell, E. E., Eakin, R. E., and Williams, R. J., *J. Am. Chem. Soc.*, **62**, 175 (1940).

⁶ Woolley, D. W., *J. Biol. Chem.*, **140**, 453 (1941).

ISOLATION OF AN ANTIBIOTIN FACTOR FROM EGG WHITE

By D. W. WOOLLEY AND L. G. LONGSWORTH

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, September 12, 1941)

Eakin, Snell, and Williams (1, 2) have shown that it is possible to prepare highly active concentrates from raw egg white which combine stoichiometrically with biotin and render it unavailable to yeast. At the time the preliminary note by Eakin *et al.* (1) appeared, it had been observed in this laboratory that egg white rendered biotin unavailable for *Clostridium butylicum* and the concentration of the injurious factor in egg white was begun, since it appeared that such a reagent would be useful in demonstrating the biotin needs of the more fastidious microorganisms (3). A substance was obtained which was homogeneous on electrophoresis and in the ultracentrifuge. During the writing of this manuscript, the second paper by Eakin and his associates (2) appeared in which they report a further concentration of the active ingredient of egg white. Although their method of purification differs somewhat from that used by us, their latest material has essentially the same biological activity as ours. Consequently, this paper confirms and, in several respects, extends their work. In particular, experimental evidence is presented as to the purity of our material.

Eakin *et al.* have named their material *avidin*. In this paper we refer to our substance as an antibiotin factor. This term may be abbreviated as AB and the compound which AB forms with biotin as BAB. Since results obtained thus far in this laboratory indicate that the phenomenon of antivitamin extends to other members of the B group, a uniform nomenclature may serve to clarify the field. Thus a substance active against thiamine, *i.e.* antithiamine, could be abbreviated AT, and the inactive complex TAT.

EXPERIMENTAL

Method of Assay—A basal medium was made by dissolving the following substances in 1 liter of water: glucose 200 gm., KH_2PO_4 8 gm., NH_4NO_3 18 gm., $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ 1.3 gm., thiamine 1 mg., riboflavin 2 mg., pantothenic acid 2 mg., nicotinic acid 2 mg., adenine 25 mg., uracil 5 mg., pyridoxine 2 mg., pimelic acid 2 mg., choline chloride 50 mg., and inositol 200 mg. 5 cc. portions of this solution were placed in 50 cc. Erlenmeyer flasks and enough water was added so that when the dilutions of the AB preparation to be assayed were added the volume would be 10 cc. Sufficient biotin methyl ester¹ was also included to give a final concentration of this material of 0.001 γ per cc.; the flasks were stoppered loosely with cotton and heated in an autoclave (15 pounds for 15 minutes). The proper dilutions of the preparation to be tested were then added, and each flask was inoculated² as described elsewhere (4). After the flasks had been incubated at 30° for 24 hours, the turbidity of the contents of each was determined quantitatively (4). The turbidity was plotted against the quantity of preparation added per cc., and the amount required to produce half maximal inhibition of growth was ascertained. This amount was said to contain an antibiotin factor unit (ABU). Egg white contained 1 ABU in 1.2 to 1.5 mg.; that is, 1.2 to 1.5 mg. of egg white combined with 0.001/2 or 0.0005 γ of biotin methyl ester.

Preparation of Antibiotin Factor—1 volume of fresh egg white was slowly added to 4 volumes of acetone which was stirred vigorously. After standing for several hours, the mixture was filtered in a press, the filter cake was ground in a meat chopper and placed in a volume of water one-third that of the original egg white, and the suspension again filtered in a press. The extract was discarded. The cake was ground and extracted with 1 per cent NaCl solution

¹ We wish to thank Dr. Vincent du Vigneaud for gifts of crystalline biotin methyl ester.

² Although yeast growth was inhibited by AB which was introduced before inoculation, when the AB was added 6 hours after inoculation (growth just barely visible), no effect was observed on the subsequent rate of growth. This suggested that the biotin of the medium was trapped in the yeast cells and passed along to the new buds internally, and that the yeast cell was impervious to AB.

equal in volume to that of the original egg white. The suspension was filtered in a press and the cake was again extracted with salt solution. The combined extracts, which contained the antibiotin factor, were filtered through paper and enough solid ammonium sulfate was added to the filtrate to half saturate it. The precipitate was filtered off and discarded, and the filtrate was treated with enough ammonium sulfate to saturate it completely. The precipitate was filtered off, washed, and dissolved in water. This solution was dialyzed against running tap water for 30 hours. The precipitate which formed was collected by centrifugation and washed repeatedly with water. The inactive filtrate was discarded. The precipitate was extracted with half saturated ammonium sulfate solution whose volume was one-tenth that of the original egg white, and the residue was filtered off and washed. The filtrate and washings were then dialyzed against running water for 30 hours. The precipitate which formed was collected in a centrifuge, and washed with water. In most instances it was dried at -40° to a fluffy white product.

The material obtained in this manner varied in potency from one preparation to another; namely, from 5 to 10 ABU per microgram. 40 to 80 per cent of the activity of egg white was recovered in such preparations. Electrophoretic examination showed that those preparations with the highest potency consisted of essentially one homogeneous component, whereas the others contained a second inert constituent which, however, could be readily eliminated by electrophoretic separation. Our purest material was therefore obtained with the latter procedure and always contained 10 ABU per microgram.

The methods of electrophoretic analysis and separation used in this research have already been described (5). The solid was suspended in one of the buffer solutions listed in Table I and dialyzed against the buffer for several days. The undissolved matter was centrifuged out and the clear solution was examined at 0° . Portions of the solutions of the electrophoretically separated components were assayed for potency. As may be deduced from the mobility data of Table I, the active material migrated toward the cathode at all pH values below 10.0, whereas the accompanying impurity, when it was present, migrated cathodically

at pH values below 4.3 and anodically above this value. Neither of these components was identical with any of the previously recognized constituents of egg white (5).

Properties of Antibiotin Factor—Many of the physical properties of AB are apparent from the manner of preparation. Thus it is insoluble in water and in saturated ammonium sulfate solution, sparingly soluble in dilute salt solutions, and rather soluble in strong salt solutions.

The preparations obtained were not obviously crystalline. As the protein precipitated during dialysis, it appeared as very small

TABLE I
Electrophoretic Mobilities of Components of Antibiotin Factor Preparation

Buffer solvent	pH at 25°	U × 10 ⁵ at 0°	
		AB	Impurity
0.1 N HCl.....	1.09	8.9	7.9
0.02 " NaAc,* 0.2 N HAc, 0.08 N NaCl.....	3.61	6.4	2.3
0.1 N NaAc, 0.02 N HAc.....	5.35	4.7	-3.0
0.02 " NaV,† 0.02 " HV, 0.08 N NaCl ..	7.84	2.2	-6.8
0.1 N NaOH, 0.12 N glycine.....	10.35	-0.3	-9.0

* Ac = acetate.

† V = diethyl barbiturate.

white particles of uniform shape and size but without definite crystal faces.

Combustion analyses showed the presence of C 45.5, H 6.6, N (Dumas) 10.8, ash 10.6. Analysis of one sample revealed 1.6 per cent phosphorous.

A solution of the antibiotin factor was very kindly examined for us in the ultracentrifuge by Dr. A. Rothen of these Laboratories. It was homogeneous and had a sedimentation constant, referred to water, of 4.7×10^{-13} at 20°. An accurate value for the molecular weight was not obtained, since the diffusion constant was not determined. If the particles are assumed to be spherical, the molecular weight would be close to 70,000, whereas it would be somewhat less than this if the molecules were not spherical.

The mobility-pH curve of the antibiotin factor was essentially straight over the pH interval studied. In order to determine whether combination with biotin affected the mobility, a solution of pure AB was added to an excess of biotin methyl ester and the mixture was dialyzed. Examination of the physiologically inactive BAB complex at pH 3.61 revealed that its mobility, 6.37×10^{-5} , was not significantly different from that, 6.45×10^{-5} , of AB. This result indicated that the size and charge of the antibiotin factor were not changed appreciably on combination with biotin and was thus consistent with our conclusion (*vide infra*) that only 1 molecule of biotin methyl ester (mol. wt., 256) combined with each molecule of AB (mol. wt. $\leq 70,000$).

The antibiotin factor is relatively stable toward changes of pH and temperature. Activity was retained when solutions of AB in dilute acetic acid were boiled. No appreciable loss of potency was observed after solutions of AB had stood at room temperature for several days or in the ice box for several weeks at pH ranging from 1.0 to 11.0. The antibiotin factor was precipitated from acid aqueous solution by picric acid and by flavianic acid, and activity could be regained from such precipitates by treating them with alcoholic ammonia.

Attempts were made to discover whether AB possessed enzyme activity. No proteinase, antitrypsin, or lysozyme activity could be demonstrated.

Specificity of Antibiotin Factor—The recognition of the high isoelectric point of AB led to the assay of other basic proteins for AB activity. Salmine had 0.2 per cent of the activity of AB; *i.e.*, 0.02 ABU per microgram. A nucleohistone prepared from liver by Dr. A. E. Mirsky of the Hospital of the Rockefeller Institute, had 0.1 per cent of the activity of AB. The inhibitory action of both of these proteins of widely separated origin was erased by additional amounts of biotin. It may be that these substances were impure and contained the same AB as hen's egg. If this is true, the presence of AB in fish and in mammals is thereby indicated. If the activity is an attribute of the pure proteins, it is difficult to envision the combination, for the low activity would require that several molecules of protein combine with 1 molecule of biotin.

DISCUSSION

The above data demonstrate that it is possible to prepare from hen's eggs a substance which is approximately 15,000 times more effective in inactivating biotin than is the egg white itself. Although not obviously crystalline, this preparation was homogeneous in the electrophoresis apparatus and in the ultracentrifuge. On the basis of these two criteria it was, therefore, a pure substance. The data regarding molecular size indicate that 1 molecule of biotin combined with 1 of the active protein. This follows from the fact that their combining weights are, roughly, in the same ratio, *i.e.* 0.005:1, as their molecular weights, *i.e.* 256:70,000. It must be remembered that the values obtained by assay involved the inherent errors of a biological test and that the true molecular weight of AB may be somewhat less than the value computed from its sedimentation constant alone.

SUMMARY

An antibiotin factor, a basic protein (isoelectric point pH 10) which combines firmly with biotin, has been isolated from egg white. The preparations were 15,000 times more active than egg white and were homogeneous in electrophoresis and sedimentation experiments. Some chemical and biological properties of the protein have been investigated. This protein is similar in biological activity to the substance isolated from egg white by Eakin, Snell, and Williams (1) and called by them avidin.

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SYNTHESIS OF INOSITOL IN MICE

By D. W. WOOLLEY, Ph.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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Since it has been shown (1, 2) that inositol possesses vitamin activity, it has been desirable to investigate the metabolism of this compound. Some studies have been made previously, but the work has been seriously limited by the lack of suitable methods of quantitative estimation. With the development of a satisfactory micromethod for the estimation of inositol (3), it has been possible to study the metabolism of this substance in some detail. In particular, it has been possible to show that while mice require inositol in the diet, they are able to synthesize the compound when none is ingested. Furthermore, the site of synthesis has been indicated, and a possible explanation for the numerous spontaneous cures of alopecia (4) has been discovered. Finally, the influence of pantothenic acid on the metabolism of inositol has been demonstrated.

EXPERIMENTAL

Method of Analysis.—Inositol determinations were made according to the method recently described by Woolley (3). Individual mice were killed with chloroform, weighed, and suspended in 50 cc. of HCl of such concentration that the final suspension was 18 per cent HCl. The mixture was refluxed for 6 hours and extracted twice with ether. The analysis for inositol was then conducted on the aqueous phase as previously described. In every case in which animals had received a diet containing inositol they were fed a ration free of this substance for 3 days before analysis. This was done in order to remove ingested inositol from the intestinal tract.

Variation in the values observed for individual male weanling mice is illustrated by the data given in Table I.

Synthesis of Inositol in Mice

In order to determine whether mice were able to synthesize inositol, the following experiments were performed.

Forty-five weanling male mice were fed a highly purified diet composed of sucrose, inorganic salts, casein, cod liver oil, corn oil, thiamin, riboflavin, vitamin B₆, nicotinic acid, choline, and pantothenic acid. The composition of this diet has been described (2).

Ten mice were individually analyzed and the average inositol content of a weanling mouse was thus obtained (see Table II). After the animals had been

fed the purified ration for 2 weeks ten more animals were analyzed. A similar group was analyzed at 4 weeks and another group of five at 6 weeks. The inositol content of an average mouse at biweekly intervals on this diet is shown in Table II. It can be seen that the mice increased in total content of inositol per mouse even though none of this substance was ingested. For purposes of comparison the average inositol content of six mice raised on stock rations for 4 weeks after weaning is included in Table II. The inositol content of six

TABLE I
Inositol Content of Individual Weanling Male Mice

Mouse No.	Live weight	Inositol content
	<i>gm.</i>	<i>gamma/mg.</i>
240	7.5	0.50
241	8.5	0.50
242	8.5	0.47
240A	8.5	0.47
345	7.5	0.48
340	7.5	0.50
341	8.5	0.50
342	8.5	0.47

TABLE II
Inositol Content of Mice Fed Rations with and without Inositol

Description of mice	Average live weight	Inositol content	Total amount of inositol
	<i>gm.</i>	<i>gamma/mg.</i>	<i>mg.</i>
Weanlings.	7.0	0.49	3.4
2 wks. without inositol.	15.0	0.35	5.3
4 wks. without inositol.	24.0	0.36	8.6
6 wks. without inositol.	35.0	0.35	12.3
4 wks. on stock ration.	25.0	0.39	9.8
4 wks. with inositol	23.0	0.54	12.4

animals fed the highly purified ration plus 100 mg. of inositol per 100 gm. of ration for 4 weeks after weaning is also shown.

The experiment was repeated with a second group of nine mice. These animals increased in inositol content on the average from 4 mg. to 12 mg. in 6 weeks. The experiment was again repeated using eight animals. These increased in total inositol content from 4 mg. to 11.4 mg.

In the presence of adequate pantothenic acid one characteristic of inositol deficiency was the variation in gain of weight which individual animals exhibited. Another characteristic was that, at about the 6th week of the experimental period, many showed a precipitous loss of weight. Unless inositol was fed when this loss of weight began, death resulted. The rate of loss was

as great as the rate of gain had been before the decline began. A mouse typical in this respect was No. 313, which gained from 9.5 gm. to 29.5 gm. in 5 weeks. In the following 10 days the weight declined to 21.5 gm. 100 mg. of inositol per 100 gm. of ration were added, and in the next 8 days the mouse increased in weight to 27.0 gm. When inositol was not administered to similar animals, death resulted after their weight had decreased to 15 to 20 gm. Death was averted only when inositol was fed soon after the loss of weight became apparent. In several cases these phenomena were not accompanied by alopecia at any stage of the experiment; and in no case was loss of hair observed during the period of precipitous loss of weight. Thus inositol deficiency was not invariably accompanied by alopecia.

The low incidence of alopecia has been reported on previous occasions (4). Examination of the data for individual mice in the first experiment described above demonstrated again that inositol deficiency cannot be produced in all animals. In contrast to the uniformity in content of inositol of weanling mice (Table I) it was found that several of the animals kept on the inositol-free ration had a low content. Thus it was low in two out of the ten analyzed after 2 weeks (0.24 and 0.27 gamma per mg., as compared to 0.35 for the average of the group); and after 4 weeks in three out of ten (0.30, 0.29, 0.31 gamma per mg.). In an independent group of seven mice three showed precipitous loss of weight after 7 weeks; in a group of five mice two behaved similarly; while in a third group of seven two lost weight as described.

Relation of Pantothenic Acid to the Synthesis of Inositol.—In order to investigate more fully the relationship of pantothenic acid and inositol to alopecia (4) the following experiments were performed. A group of twenty-two weanling mice were fed the above ration, minus pantothenic acid. Ten animals were analyzed at the beginning of the experiments. The remaining twelve were analyzed 3 weeks later. The average content of inositol in the first group was the same as that previously recorded in Table II for weanling mice (0.49 gamma per mg.; 4.0 mg. per mouse). The average values in the second group were 0.24 gamma per mg. and 4.3 mg. per mouse. Thus the inositol content per unit weight decreased and the total amount of this substance in the mouse remained approximately the same.

The experiment was repeated twice with groups of six mice with the same result as in the first trial. In a fourth experiment with 12 mice the content of inositol did not decrease significantly (0.48 to 0.41 gamma per mg.) in 2 weeks, undoubtedly because of the small gain in weight of the group (from 6.5 gm. to 10 gm.). That is, the quantity of inositol contained in a mouse at the start and presumably carried through the test period was not distributed in as much tissue as in the other experiments.

Addition of pantothenic acid to the diet of animals deficient in this vitamin resulted in the synthesis of inositol. A typical experiment was as follows:

A group of fifteen mice were fed the ration deficient in pantothenic acid for 2 weeks. The average content of inositol at the beginning was 0.49 gamma per mg. and the total amount in the average mouse was 4 mg. After the mice had been deficient in pantothenic acid for 2 weeks the content of inositol had decreased, but the total amount in a mouse was the same as at the beginning (judged from the average of two mice analyzed). Pantothenic acid was then restored to the diet. Two to 3 weeks after the restoration the typical alopecia of inositol deficiency made its appearance in seven of the mice. Four out of the seven hairless individuals exhibited spontaneous cure of the alopecia 2 to 3 weeks after its appearance even though no inositol was fed. Analyses of these spontaneously cured animals revealed that in them inositol had increased to normal values (0.39 gamma per mg.; 11.7 mg. per mouse). Analyses of those which remained hairless showed that they had not increased markedly in content of inositol.

Microbial Synthesis of Inositol

In an effort to discover the reason for the spontaneous cures of alopecia which were observed (4, and above) the following experiments were performed:

The intestinal tract of a mouse which had exhibited spontaneous cure of alopecia was removed aseptically and placed in 10 cc. of a synthetic medium. This medium had the composition described by Woolley (5), except that inositol and thioglycollic acid were omitted. It was thus a highly purified mixture of all available growth factors and of glucose, inorganic salts, and amino acids. The tube was incubated for 24 hours at 37°, and then a drop of the suspension was introduced into a second 10 cc. of medium of the same composition. After 24 hours incubation, 1 cc. of this passage of culture of organisms from the intestinal tract was added to 500 cc. of medium of the same composition. Incubation at 37° was continued for 60 hours. The cells were then collected by centrifugation, hydrolyzed, and analyzed for inositol. The metabolism solution was concentrated under reduced pressure and similarly analyzed.

As is shown in Table III, the organisms obtained by passage of a culture from the intestinal tract synthesized inositol and at least 80 per cent of the amount formed was retained in the cells. The experiment was repeated with the same mixed culture (which had been stored at 0°) for an incubation period of only 16 hours. Approximately the same quantity of inositol was formed as previously. Hence the incubation periods in subsequent tests were 16 hours in length.

The intestinal tract of a mouse from the same group of animals which had lost its hair and had not exhibited spontaneous cure was treated similarly. The bacteria from this mouse showed a much smaller content of inositol (Table III). The experiments were repeated on two mice from a second run, one of which had become hairless but had then exhibited a spontaneous cure and the other of which had remained hairless. The culture of organisms from the first mouse synthesized 0.42 gamma of inositol per cc. of culture and that from the second mouse formed 0.12 gamma. A third experiment with two more mice, cured and hairless like the others, was performed in the same manner and it was found that the values were 0.40 and 0.0 gamma per cc. for the

culture from the spontaneously cured mouse and the hairless mouse respectively. In this case the hairless mouse was examined as soon as alopecia developed, while in the other instances animals were taken which had been hairless for about one week.

It has been found that the synthesis by the cells of the culture was not influenced by gramicidin and that a prominent intestinal inhabitant did not form inositol. One of the cultures already tested, which was procured from a mouse which had exhibited spontaneous cure of alopecia, was inoculated into a medium of the composition described above, to which had been added 10 gamma per cc. of crystalline gramicidin.¹ A control test was done, using the same culture without the addition of gramicidin. The cells from each medium were collected and analyzed for inositol. As can be seen from Table III gramicidin did not

TABLE III
Inositol Content of Cultures of Intestinal Organisms

The numbers in parentheses indicate the length of the period of incubation.

Material analyzed	Inositol content of culture
	<i>gamma/cc.</i>
Cells of culture from spontaneously cured mouse (60 hrs.) . . .	0.40
Supernatant fluid after centrifugation of the above cells . .	Less than 0.1
Cells of mixed culture from above mouse (16 hrs.)	0.38
Cells from the above culture grown in gramicidin	0.38
Cells of mixed culture from hairless mouse	0.18
Cells of <i>E. coli</i>	Less than 0.1

influence the synthesis. The cells of one of the most important bacterial forms in the intestinal tract of the mouse, *Escherichia coli*, as grown in the purified medium, were analyzed and found not to contain inositol in demonstrable amounts (Table III).

Influence of Pantothenic Acid on Inositol Metabolism

The occurrence of alopecia in a high percentage of the mice to which pantothenic acid had been restored after 2 or 3 weeks of deficiency has been mentioned above. It seemed likely that if inositol were fed from the beginning, this delayed appearance of alopecia might be prevented. A group of ten mice were fed the basal ration referred to throughout this paper, from which pantothenic acid was omitted and which was supplemented with 100 mg. of inositol per 100 gm. After 2 weeks pantothenic acid was restored and inositol was omitted. Two to 2½ weeks after this change typical alopecia developed in all but three of the animals. Analysis of three of the hairless mice showed that they contained little more inositol than did weanling mice (6.4 mg. compared to 4.5 mg.).

¹ We wish to thank Dr. R. J. Dubos for gifts of gramicidin.

Similarly, it had been found that two of the animals at the time that pantothenic acid was added were deficient in inositol even though they had received it in the diet (4.0 mg. compared to 4.4 mg.).

The experiment was repeated with the following modifications. A group of six mice were fed the ration which contained inositol but no pantothenic acid. After 2 weeks analysis of three mice again showed that they were deficient in inositol. The other three mice were continued on inositol for 3 days following the addition of pantothenic acid. Analyses of these revealed a normal content of the vitamin (0.42 gamma per mg.). Thus it appeared that when pantothenic acid was absent from the ration inositol deficiency developed even though this substance was ingested. Addition of pantothenic acid to the diet restored the inositol content of the mice to normal within 3 days.

TABLE IV

Distribution of Free and Total Inositol in Various Natural Products

Except in the case of the extracts analyses were based on weights of undried samples.

Material analyzed	Free inositol	Total inositol
	<i>gamma/mg.</i>	<i>gamma/mg.</i>
Normal mice (6 individuals)	0.25	0.41
Inositol-deficient mice (3 individuals)	0.20	0.30
Beef skeletal muscle	0.55	0.88
Beef brain	3.0	6.0
Beef pancreas extract	1.2	5.0
Aqueous alcohol extract of rice bran	0.37	0.53
Dialyzed rice bran extract	0.0	0.16

Free and Combined Inositol

Since it has been shown (6) that yeast is unable to respond to inositol esters, it was thought possible that the method for the estimation might be refined to differentiate free from combined inositol. It has been shown (2) that liver contains a non-dialyzable, water-soluble substance which liberates inositol when treated with acid or alkali. It has been found that yeast does not respond to this combination. This has made possible the analysis of natural products for free inositol, separate from total inositol. The difference between free and total represents combined inositol.

For the analysis of mice for free inositol the following procedure was used.

A mouse was ground and suspended in water. An aliquot of this suspension was hydrolyzed with HCl and total inositol was determined in the hydrolysate as previously described. A second aliquot of the suspension was heated in an autoclave (15 pounds for 15 minutes), centrifuged, and the precipitate washed with water. Direct analysis of this liquid without acid hydrolysis gave a value for free inositol. Since the combined inositol present in liver extract, brain extract, pancreas extract, and rice

bran extract was not rendered available to yeast by autoclaving, this procedure was used to coagulate proteins and destroy enzymes.

By this technique approximately 60 per cent of the inositol content of normal mice was found to be free. Similarly, 50 per cent of the total inositol content of brain and 60 per cent of that in skeletal muscle was found to be free. The proportion of free inositol in normal mice did not differ significantly from that in deficient animals. Some representative data are shown in Table IV. In the case of tissues the value for free inositol represents the amount which can be extracted and does not include any free inositol which may be retained in insoluble residues.

DISCUSSION

It can be clearly seen from the data in Table II that mice synthesized inositol when fed the purified ration. It appears that the presence of pantothenic acid in the ration was of importance for this synthesis. Those animals which did not receive pantothenic acid failed to increase markedly in total inositol content even though they did gain in weight; whereas, when pantothenic acid was added to the ration, the inositol content of the mice increased. It may be of interest to note that in no case of deficiency did the total amount of inositol in a mouse decrease even though the content per unit weight did fall. Hence it was necessary that the animals should grow if the inositol deficiency was to be recognized by a decreased content of the vitamin per unit weight.

One site of inositol synthesis appears to be the intestinal tract. When a mouse exhibited spontaneous cure of alopecia, a simultaneous increase in the total content of inositol took place. Organisms cultivated from the intestinal tract of mice which had exhibited spontaneous cure synthesized inositol in the instances tested and to a much greater extent than did the organisms isolated by the same method of cultivation from hairless mice. Since the synthesis took place in the presence of gramicidin, which inhibits the growth of Gram-positive bacteria (7) and since the gramicidin-treated cultures on microscopic examination were found to be Gram-negative, it is probable that the organisms responsible for the synthesis were Gram-negative. However, a prominent Gram-negative organism of the intestinal tract, *E. coli*, did not form inositol. Since inositol deficiency could be produced regularly in a small percentage of mice fed a deficient diet, it must be concluded that the inositol synthesized by such organisms was not sufficient to meet all of the requirements of the mouse, or else that, under the conditions studied, the organisms did not become established in all animals. This latter hypothesis may explain more adequately why only some animals on an inositol-free diet develop alopecia. The data show further that the metabolism of inositol is influenced by pantothenic acid. Mice did not increase in total content of inositol even when this sub-

stance was present in the diet unless pantothenic acid was also present. It has not been established whether this phenomenon was due to failure of absorption or to some more obscure metabolic disturbance.

SUMMARY

It has been shown that mice are able to synthesize inositol. This synthesis was not observed when pantothenic acid was absent from the diet. Cultures from the intestinal tract of animals which exhibited spontaneous cure of alopecia yielded microorganisms which synthesized much more inositol than did organisms isolated in the same fashion from the tracts of mice that had become hairless. Some observations on the distribution of free and combined inositol have been made and it has been shown that several biological materials contain combined inositol. It has been found that deficiency of inositol can develop even when inositol is present in the diet if pantothenic acid is omitted.

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ON THE FORMATION OF CHYMOTRYPSIN FROM CHYMOTRYPSINOGEN

By J. A. V. BUTLER¹

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton,
New Jersey)

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The conversion of chymotrypsinogen into chymotrypsin by trypsin was discovered by Kunitz and Northrop² and shown to be a simple unimolecular catalytic reaction. In connection with the more extended study of the kinetics of trypsin action reported in the following paper, the activation energy of this reaction was required. Determinations of the velocity constant have been made at several temperatures (see Table I and Fig. 1), from which it is found that the activation energy has the comparatively high value of 16,300 calories. The reaction is abnormally rapid for this energy and the significance of this fact is discussed in the following paper.

Some experiments designed to elucidate the nature of the reaction also were carried out. There is an increase in the formol titration in the course of the reaction (Fig. 2) which parallels the growth of enzymatic activity at the beginning but continues to increase slowly when the conversion is completed. It follows that the conversion is accompanied by a small amount of some secondary action, as has also been found in other experiments.² The primary increase of the formol titration is most simply interpreted as being due to the liberation of acid groups produced by the splitting of peptide bonds.

The number of peptide bonds split can be estimated in two ways. (1) The increase of formol titration during the activation amounts to about 7.5×10^{-6} equivalent in a solution containing 1.3×10^{-6} g. mole per ml. of chymotrypsinogen, or about 6 equivalents per g. mole, *i.e.*, the conversion produced 6 acid groups in the molecule. (2) The initial rate of hydrolysis as measured by the formol titration can be compared with the rate of formation of chymotrypsin molecules. The velocity constant of the reaction was $2.0 \times 10^{-2} \text{ min.}^{-1}$, *i.e.*, at the outset $2.0 \times 10^{-2} \times 1.3 \times 10^{-6} = 2.6 \times 10^{-8}$ g. mole per ml. of chymotrypsinogen is converted initially per minute, while the initial rate of increase of the formol titration is 10^{-7} equivalent per minute. The conversion of each molecule thus involves 4 equivalents. The two

(1) Fellow of the Rockefeller Foundation.

(2) M. Kunitz and J. H. Northrop, *J. Gen. Physiol.*, **18**, 433 (1935).

TABLE I

Velocity Constants of Conversion at Various Temperatures

Reaction mixture: 7 ml. $\text{M}/15$ phosphate buffer, pH 7.5; 1 ml. dialyzed chymotrypsinogen solution (total nitrogen = 1.26 mg. per ml.); 1 ml. trypsin solution containing 0.0111 mg. protein nitrogen per ml. Hemoglobin activity = $0.00118 [\text{T.U.}]_{\text{ml.}}^{\text{Hb}}$; taking specific activity of pure trypsin as $0.16 [\text{T.U.}]_{\text{mg.}}^{\text{Hb}}$, the purity of trypsin was 0.67%. Concentration in solution added is 0.046 mg. trypsin per ml., or taking the molecular weight of trypsin as 36,500 = 1.27×10^{-9} mole per ml. The concentration of trypsin in the reaction mixture was 1.4×10^{-7} mole per liter.^a

	0°C.	6.3°C.	13.0°C.	19.6°C.
$k \text{ min.}^{-1}$	3.35×10^{-3}	5.7×10^{-3}	13.4×10^{-3}	24.3×10^{-3}
$k \text{ sec.}^{-1}$ for 1 mole trypsin per liter.....	3.97×10^2	6.8×10^2	15.9×10^2	29.0×10^2

^a I am greatly indebted to Dr. Margaret R. McDonald for carrying out the trypsin assay quoted here.

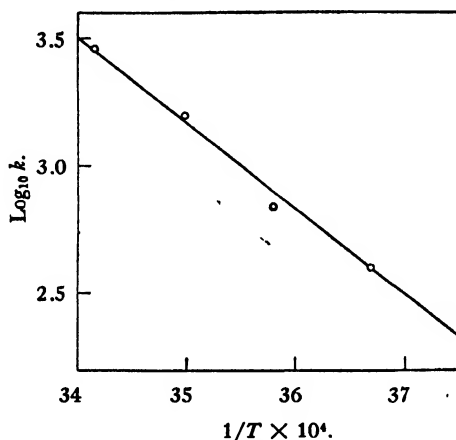
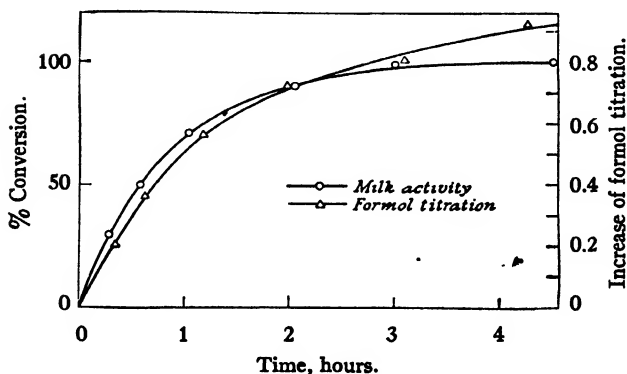
FIG. 1. Action of trypsin on chymotrypsinogen ($\log k$ against $1/T$).

FIG. 2. Increase of formol titration during activation. Composition of reaction mixture: 6 ml. chymotrypsinogen (9.1 mg. protein N/ml.); 1 ml. pH 7 buffer; 0.5 ml. trypsin solution (0.1 mg. N/ml.); temperature, 13.5° .

methods therefore lead to the conclusion that, in the formation of a chymotrypsin molecule, from 4 to 6 equivalents of acid are liberated.

There is, however, no evidence that any appreciable quantity of nitrogenous material is split from the chymotrypsinogen molecule in the course of the reaction. Kunitz and Northrop² found that a slow increase in the amount of

TABLE II

Rate of Reaction in Presence of Deuterium Oxide

Reaction mixture: 8 ml. water or 70 per cent. deuterium oxide; 0.5 ml. of chymotrypsinogen solution, 0.5 ml. 1 M pH 7.5 phosphate buffer, 0.2 ml. trypsin (0.05 mg. nitrogen per ml.).

Time, minutes.....	30	60	90	120	160
Per cent. conversion water.....	30.5	68	76	82	85
Per cent. conversion deuterium oxide..	41.5	69	74	78	83

TABLE III

Effect of Concentration of Chymotrypsinogen on Velocity Constant

Reaction mixture: 4.5 cc. chymotrypsinogen solution in M/10 pH 7.5 buffer, 0.5 cc. trypsin (approximately 0.01 mg. nitrogen per ml.) 25°.

<i>c</i> , mole per liter.....	1.32×10^{-3}	0.15×10^{-3}	0.015×10^{-3}
<i>k</i> , hours ⁻¹	0.42	0.58	0.58

TABLE IV

Formation of Non-Protein Nitrogen in Conversion of Chymotrypsinogen to Chymotrypsin

Reaction mixture: 10 ml. dialyzed chymotrypsinogen solution containing 3.68 mg. nitrogen per cc.; 0.5 ml. trypsin, approximately 1 mg. nitrogen per cc.

Time, hours	Conversion, %	Non-protein nitrogen, %
0	0	3.9
1	80	4.5
2	90	3.7
23	100	6.3
47	—	10.6
71	—	10.4

non-protein nitrogen accompanies the reaction and continues when the conversion is completed. They regarded this as being mainly formed by a side reaction, leaving open the question whether any part of it was directly associated with the formation of chymotrypsin. This experiment was repeated using a much higher concentration of trypsin, so that the conversion was completed in less than an hour (Table IV). The increase in the amount of "non-protein" nitrogen during the conversion was certainly less than 1% of the total nitrogen present, the accuracy of the determination being of the order of 0.5%. The increase to be expected, if each peptide bond broken

releases only 1 nitrogen atom, is from 1 to 1.5%. It is therefore probable that no molecules containing nitrogen are split off.

Since trypsin hydrolyzes the terminal amide group of benzoyl-arginine amide,³ it appeared to be possible that its action causes the hydrolysis of one or more amide groups. It was found that the amount of free ammonia liberated in the conversion was negligible (Table V).

TABLE V

Liberation of Free Ammonia in Formation of Chymotrypsin

Reaction mixture: 30 ml. dialyzed chymotrypsinogen solution containing 10 mg. nitrogen per ml. at pH 7.5, 1 ml. trypsin, 0.1 mg. nitrogen per ml. The activation was practically complete in 0.5 hour.

Time, hours	Free NH ₃ distilled from 5 ml. as mg. nitrogen
0	0.016
0.5	.026
18	.020
66	.030

TABLE VI

Effect of Salt Concentration on the Reaction Velocity

Composition of reaction mixture: chymotrypsinogen solution 1 ml., $M/2$ pH 7.5 phosphate solution 1 ml., salt solution of appropriate strength 6 ml., trypsin (0.01 mg. nitrogen per ml.) 1 ml.

Salt concn., N	Relative velocity constants		
	KCl	NaCl	Na ₂ SO ₄
0	1.0	1.0	1.0
0.5	0.25	0.50	0.63
1.0	.10	0.26	.53
1.5	.08	—	.44
2.0	—	—	.54

It is possible, of course, that an acid containing no nitrogen is liberated, but no acids of this kind have been detected in proteins, and it is much more probable that the reaction is the opening of peptide bonds in closed rings. Very little information about the mode of action of proteolytic enzymes has been obtained. The fact that the rate of the activation is completely uninfluenced when the hydrogen in the solvent is largely replaced by deuterium (Table II) shows that the reaction is not similar to a hydrogen ion hydrolysis and does not involve protons in any direct way. This is in contrast to the behavior of emulsin on glucosides.⁴

(3) M. Bergmann, J. S. Fruton and H. Pollok, *J. Biol. Chem.*, **127**, 643 (1939).

(4) K. F. Bonhoeffer, *Erg. der Enzymforschung*, **6**, 47 (1937).

Kunitz and Northrop found that the velocity constant of the reaction was independent of the chymotrypsinogen concentration up to 0.42 mg. of protein nitrogen per ml. (8×10^{-5} mole per liter). To find whether a stable complex exists between the enzyme and the substrate, determinations were made at still higher concentrations and it was found (Table III) that the velocity constant was only 20% smaller at a chymotrypsinogen concentration of 1.3×10^{-3} mole per liter than at low concentrations. This diminution might be due to secondary effects arising from the increased concentration rather than approaching saturation of a complex; but it is clear that the Michaelis constant is greater than 1.3×10^{-3} mole per liter.

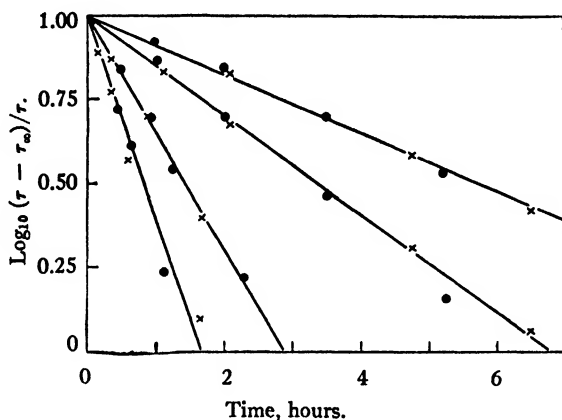


FIG. 3. Conversion of chymotrypsinogen into chymotrypsin at various temperatures (two distinct experiments at each temperature).

Some experiments were made (Table VI) on the effect of salts on the rate of the reaction.⁵ All the salts tried depress the rate, the effect being in the order $\text{KCl} > \text{NaCl} > \text{Na}_2\text{SO}_4$ for equal normalities. Up to 1 *N*, the logarithm of the rate varies roughly linearly with the salt concentration, but at higher concentrations the change is smaller and with sodium sulfate the rate reaches a minimum and begins to rise again. This effect is probably produced by the salting-out action of the salt on the reaction complex. If the reaction complex between the chymotrypsinogen and the trypsin is salted-out to a greater extent than the two constituents, this behavior would occur. The reaction complex is probably negatively charged since the experiments were made at a *pH* at which chymotrypsinogen is negatively charged, while trypsin is in the vicinity of its isoelectric point. The marked difference between potassium and sodium salts indicates a marked cation effect, which might be expected for a negative complex.

(5) Cf. M. R. McDonald and M. Kunitz, *J. Gen. Physiol.*, **25**, 53 (1941).

EXPERIMENTAL

Determination of Rate of Activation.—The amount of chymotrypsin formed was determined by its milk clotting action using Herriott's method of observing the clotting time.⁶ The concentration of chymotrypsin is taken as inversely proportional to the clotting time τ . If τ_{∞} is the final clotting time, the fraction of chymotrypsinogen converted at any time is τ_{∞}/τ . The velocity constant was obtained by plotting $\log (\tau_{\infty} - \tau)/\tau$ against time (Fig. 3). The two runs which were carried out at each temperature were in good agreement with each other and gave close to a linear relation in this plot.

Determination of Non-protein Nitrogen.—One ml. of a suitable dilution is added rapidly to 10 ml. of 5% trichloroacetic acid at 95–100° and the mixture cooled in a water-bath at 25° and filtered. The nitrogen present in an aliquot part of the filtrate was determined by a semimicro Kjeldahl method.

Determination of Free Ammonia.—Basic phosphate is added to 5 ml. of the solution until just pink to phenolphthalein and then 5 ml. of saturated borax is added.⁷ The solution is distilled for fifteen minutes under reduced pressure at 40–42° into boric acid containing 0.1% brom cresol green. This was titrated back to a standard color by N/70 hydrochloric acid.

Formol Titration.—To 1 ml. of the solution add 0.5 ml. of 40% formaldehyde and titrate with 0.01 N sodium hydroxide to the first definite pink color. In cases where the "initial" titer is large, 1 ml. of a more concentrated alkali, which brings the initial solution into the vicinity of the end-point, is added to each sample.

SUMMARY

1. From the velocity constants of the conversion of chymotrypsinogen into chymotrypsin by trypsin at four temperatures, the activation energy of the reaction is found to be 16,300 calories.

2. The increase in the formol titration in the reaction corresponds to a splitting of 4 to 6 peptide bonds. Since very little non-protein nitrogenous material and practically no ammonia are liberated in the reaction, it is probable that the reaction is the opening of peptide bonds in ring structures.

3. The effects of some inorganic salts, of substituting deuterium oxide for water in the solvent, and of chymotrypsinogen concentration on the reaction rate have been observed.

(6) R. M. Herriott, *ibid.*, **21**, 501 (1938).

(7) G. Pucher, H. B. Vickery and C. S. Leavenworth, *Ind. Eng. Chem., Anal. Ed.*, **7**, 152 (1935).

THE MOLECULAR KINETICS OF TRYPSIN ACTION

By J. A. V. BUTLER*

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton,
New Jersey)

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Very little reliable information exists about the absolute rates and activation energies of enzyme reactions. In many cases in which measurements have been made, the reaction is sensitive to the hydrogen ion concentration and corrections for the effect of temperature on the pH are necessary before the true activation energy can be known. The object of this work was to determine these quantities for some typical proteolytic processes on both natural and synthetic substrates. Trypsin is particularly suited to a study of this kind because the same enzyme brings about a number of different reactions, *e. g.*, (a) the hydrolysis of ammonia from the synthetic peptide benzoyl-*l*-arginine amide¹; (b) the conversion of chymotrypsinogen into chymotrypsin²; (c) the autocatalytic conversion of trypsinogen into trypsin³; (d) the digestion of native and denatured proteins. In the region of the optimum pH the effect of changes of pH is comparatively small and the effect of temperature change on the pH can probably be neglected in finding the activation energy of the reaction.

It was known that good unimolecular velocity constants, which are proportional to the trypsin concentration, are obtained for reactions (a) and (b). The action of trypsin on a protein is to be regarded as a group of simultaneous or consecutive reactions and it is not usually possible to find a velocity constant covering the whole course of the reaction. Northrop⁴ showed that in the action of trypsin on casein the first stage could be distinguished by observing the change of viscosity and a fairly good velocity constant was calculated therefrom. For the comparison of reactions (a) and (b) and (d) we have used as the velocity constant of the latter the initial rate of digestion (measured by the number of acid groups liberated as determined by the formol titration),

* Fellow of the Rockefeller Foundation.

(1) M. Bergmann, J. S. Fruton and H. Pollok, *J. Biol. Chem.*, **127**, 643 (1939); K. Hofmann and M. Bergmann, *ibid.*, **138**, 243 (1941).

(2) M. Kunitz and J. H. Northrop, *J. Gen. Physiol.*, **18**, 433 (1935).

(3) M. Kunitz and J. H. Northrop, *ibid.*, **19**, 991 (1936); M. Kunitz, *ibid.*, **22**, 293 (1939); *Enzymologia*, **7**, 1 (1939).

(4) J. H. Northrop, *J. Gen. Physiol.*, **16**, 339 (1932).

divided by the molecular concentration of the substrate. This would be identical with the true velocity constant of the reaction when each molecule of substrate gives rise to one equivalent of acid on complete hydrolysis. In other cases it seems a good basis of comparison (on the assumption that the action of trypsin is really primarily the breaking of peptide bonds) as it gives the number of bonds broken by a given concentration of enzyme with equal numbers of substrate molecules. If the protein contains different kinds of bonds which are acted on by the enzyme at different rates, the observed rate will be the sum of the rates of the various actions. In many cases, however, the observed rate would be predominantly that of one particular action and no great error will arise from treating it as such. Considerable caution is required however in the interpretation of the activation energy in such a case since the different processes may have different temperature coefficients. In such a case the Arrhenius equation will not hold.

TABLE I

Enzyme	Substrate	Log ₁₀ <i>k</i> (0°)	Δ <i>H</i>	Δ <i>S</i>
Trypsin.....	Benzoyl- <i>L</i> -arginine amide	0.40	14,900	-6.2
Trypsin.....	Chymotrypsinogen	2.6	16,300	+8.5
Trypsin.....	Sturin	3.33	11,800	-4.7
Chymotrypsin....	Benzoyltyrosylglycyl amide	1.57	10,500	-17.4
Chymotrypsin....	Pepsin	2.34	11,200	-11.5
(Hydrogen ion)...	Acetyl glycine	-6.47 (60°)	21,200	-24.8

The procedure can be applied only to the initial stage of the reaction where the protein is mainly intact. As the reaction proceeds new bonds may become accessible to the enzyme, the velocity constants may be influenced by the changes which have taken place in other parts of the molecule, as is well known⁵ the products of the digestion may exert an inhibitory effect and when the protein breaks up the substrate concentration becomes indefinite.

The choice of a protein substrate presented some difficulty. It is known that trypsin frequently does not act appreciably on native proteins, *e. g.*, egg albumin, but it digests them rapidly when denatured. Casein is acted on rapidly, but is a mixture of proteins. We tried pepsin denatured by warming at pH 7.5 as a substrate; trypsin appears to have a real action on it but the amount is so small that the initial rate is very difficult to measure. Trypsin also digests protamines very easily⁶ and sturin was found to be a very suitable substrate. Experiments were also made with chymotrypsin acting on the synthetic peptide benzoyl-*L*-tyrosyl-glycyl amide⁷ and on denatured pepsin.

(5) J. H. Northrop, *ibid.*, **4**, 487 (1922).

(6) E. Waldschmidt-Leitz and T. Kollmann, *Z. physiol. Chem.*, **166**, 262 (1927).

(7) M. Bergmann and J. S. Fruton, *J. Biol. Chem.*, **118**, 405 (1937); **124**, 321 (1938).

DISCUSSION

The evidence previously available appeared to indicate that enzyme reactions are frequently abnormal kinetically. In a review of the available information⁸ Stearn found that in most cases the entropy of activation of enzyme reactions was considerably more negative than that of the same reactions catalyzed by acids, *etc.* This means that the increase in the reaction rate brought about by the enzyme is not so great as would be expected from the decrease of activation energy, so that there is a factor which might be a stringent condition of mutual orientation of the substrate and enzyme molecules which "interferes" with the reaction.

This conclusion is not borne out by the data obtained here. Figure 1 shows that over the range of temperatures studied the Arrhenius expression holds within the experimental error. Table I summarizes the characteristics of all the reactions and gives the entropy of activation, ΔS , calculated by

$$k = \frac{RT}{N_0 h} e^{-\Delta H/RT} e^{\Delta S/R}$$

or, for 0°

$$k^0 = 5.7 \times 10^{13} e^{-\Delta H/RT} e^{\Delta S/R}$$

The activation energies of these reactions are rather high for enzymatic processes (10–16 kcal.), although considerably lower than that of the hydrolysis of the peptide bond by acids which is about 21 kcal.⁹ The entropies of activation are, with one exception, reasonably close to the range (–5 to –10) usually taken as representing normal reactions.

The rates are, in fact, not very different in most cases from that calculated on the simple collision theory, *viz.*

$$k = Ze^{-\Delta H/RT}$$

where Z , the number of collisions between the reacting molecules per cc., is given by

$$Z = n_1 n_2 \left(\frac{\sigma_1 + \sigma_2}{2} \right)^2 \left\{ 8\pi RT \left(\frac{1}{M_1} + \frac{1}{M_2} \right) \right\}^{1/2}$$

where n_1 , n_2 are the numbers of the reacting molecules per cc.; $\sigma_1\sigma_2$ their diameters and M_1 , M_2 their molecular weights. Taking for trypsin $\sigma_1 = 6 \times 10^{-7}$ cm., $M_1 = 36,000$, we find that the calculated rate, which is not much influenced by the size of the substrate molecule, corresponds to $\Delta S = -6$ to

(8) A. E. Stearn, *Ergebn. der Enzymforschung*, **7**, 1 (1938).

(9) A. I. Escolme and W. C. M. Lewis, *Trans. Faraday Soc.*, **23**, 651 (1927).

—8. The conversion of chymotrypsinogen, which has a positive entropy, is considerably faster than the other reactions when allowance is made for its high activation energy.

In reactions involving one or two large molecules it can hardly be expected that every collision having the necessary energy will lead to reaction, since there must inevitably be steric or orientational factors tending to reduce the

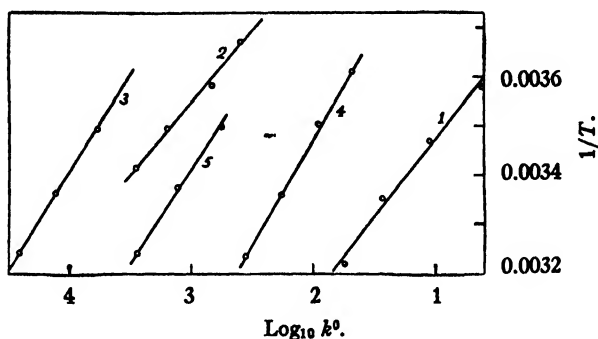


FIG. 1. Plots of $\log_{10} k^0$ against $1/T$ for: (1) trypsin on benzoyl-*L*-arginine amide; (2) trypsin on chymotrypsinogen; (3) trypsin on sturin; (4) chymotrypsin on benzoyl-tyrosyl-glycylamide; (5) chymotrypsin on denatured pepsin.

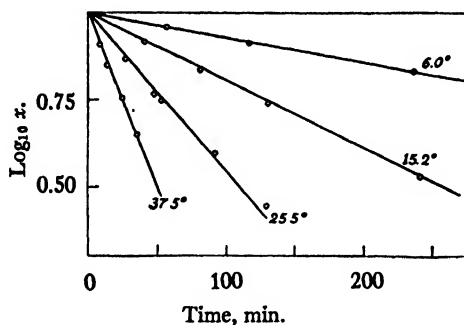
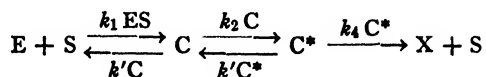


FIG. 2. Hydrolysis of benzoyl-*L*-arginine amide by trypsin

rate. It follows that even for reactions coming within the normal range for small molecules, there must be a factor producing an enhanced rate. Such a factor may be the complex formation between enzyme and substrate which is a very general feature of enzyme reactions. The formation of a stable complex will evidently increase the rate above that calculated by the collision theory, because, if the molecules spend an appreciable time in union, the chance of reaction is obviously greater than if they separate immediately on collision.

The effect on the reaction velocity of the formation of stable complexes

between the reactants does not appear to have been considered closely. If the course of the reaction is formulated as



where C is the stable complex between enzyme and substrate and C^* the "activated" complex, which gives rise to the reaction products, we can assume

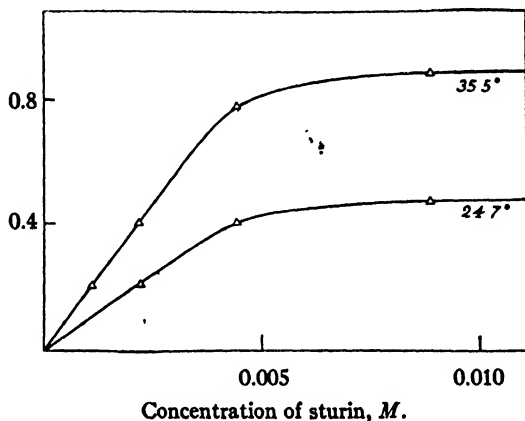


FIG. 3. Effect of substrate concentration on the rate of hydrolysis of sturin by trypsin: ordinate represents increase of formol titration, equiv. $\times 10^{-7}$, per ml. per min.

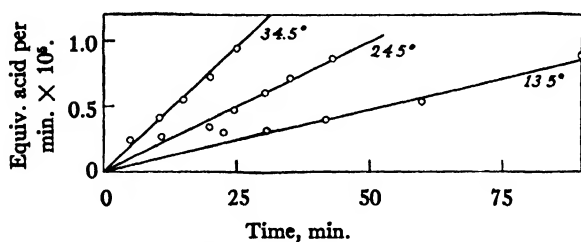


FIG. 4. Initial stages of digestion of sturin by trypsin

that C is present at an equilibrium concentration (this implies that $k_2 < k'_2$). There are now two cases: (1) The complex C^* is also present in an equilibrium concentration which will be the case if $k_4 < k'_2$. It can, of course, be assumed that the concentration of the energy-rich complex C^* is very small. The rate of reaction will be unaffected by the concentration of C , since the equilibrium concentration of C^* will be the same whether it is derived directly from E or S or from C . (2) If $k_4 > k'_2$ the activated state will not reach an equilibrium

concentration and the rate of the reaction will be k_2C , which is the rate of formation of C^* . In this case the concentration of the stable complex may be expected to be a significant factor.

It is not, however, possible at present to make any correlation between reaction rates and the complex stability which is indicated by the Michaelis constant. The formation of a complex has been demonstrated in the action of trypsin on sturin (Fig. 3), where the Michaelis constant is of the order of 7.5×10^{-8} mole per liter. In the case of trypsin on chymotrypsinogen the constant is $> 1.3 \times 10^{-3}$, and it follows that the stability of the latter complex is not so much greater than the former as to account by itself for the greater rate of the latter reaction.¹⁰ A great deal more data will have to be collected before the connection can profitably be discussed in detail.

EXPERIMENTAL

Trypsin on Benzoyl-L-arginine Amide.—The course of the reaction was followed by a modified formol titration. Five-tenths of a milliliter of the sample was added to 0.5 ml. of 40% formaldehyde with 5 drops of 0.1% phenolphthalein and titrated with 0.01 *N* aqueous sodium hydroxide. If θ_0 , θ_t , θ_∞ are titers at times 0, t , and ∞ , the fraction hydrolyzed at time t is $x = (\theta_t - \theta_0)(\theta_\infty - \theta_0)$. Figure 2 shows $\log x$ plotted against t . From the slopes of these lines we obtain the constants in Table II.

Trypsin on Sturin.—The rate of hydrolysis was followed by the modified formol titration described above. Since the initial quantity of alkali required is comparatively large, a stronger solution was made up of a strength such that 1 cc. was just insufficient for the initial titer. This quantity was added to every sample. The initial rate of hydrolysis is proportional to the sturin concentration but becomes independent of it at concentrations above about 0.0075 *M* (Fig. 3). The temperature coefficient was determined at a sturin concentration of about 2×10^{-3} *M*, taking molecular weight as 3550¹¹. The increase of formol titration with time in the first stages of the reaction is shown in Fig. 4.

Chymotrypsin on Benzoyltyrosylglycylamide.—The substrate solution contained approximately 2 mg. of the substance per ml. The reaction was also followed by the formol titration. Only a small quantity of the substrate was available, sufficient for determinations of the initial and final titers and that of one or two intermediate points, which were taken in the region of half change. From these the period of half change was estimated. The accuracy of this procedure is inferior to that of the other data in this paper and the activation energy calculated from the temperature coefficient is to be regarded as an approximation.

Chymotrypsin on Denatured Pepsin.—The pepsin was a crystalline specimen for which I am indebted to Dr. R. M. Herriott. A solution was dialyzed until practically free from salt and then 1 *M* K_2HPO_4 was added until the pH was near 7.5. This solution, which contained 7.2 mg. of nitrogen per ml., was warmed at 60° for ten minutes. The initial stage of the reaction was followed by the formol titration.

(10) The stability of the complex is inversely proportional to the Michaelis constant.

(11) K. Felix and A. Lang, *Z. physiol. Chem.*, **188**, 96 (1930).

TABLE II

Reaction mixture: 5 ml. 0.05 *M* benzoyl-L-arginine amide, 1 ml. 0.1 *M* phosphate buffer, pH 7.8; 1 ml. of trypsin solution containing 0.281 mg. trypsin nitrogen per ml. (as determined by hemoglobin activity). Concentration of trypsin in reaction mixture 6.9×10^{-6} mole per liter.

$T, ^\circ\text{C}.$	6.0	15.2	25.5	37.5
$k, \text{min.}^{-1} \times 10^3$	1.68	4.62	11.2	23.0
$k \text{ sec.}^{-1}$ for 1 mole trypsin per liter.....	4.06	11.2	27.0	55.6
$k^0 = 2.54$	$\Delta H = 14,900$			

TABLE III

Reaction mixture: 1 ml. sturin solution, 0.01 *M*, 3 ml. 0.1 *M* phosphate buffer, pH 7.5; 0.5 ml. trypsin solution containing 3.2×10^{-2} mg. per ml. trypsin as determined by rate of hydrolysis of benzoyl-arginine amide.

$T, ^\circ\text{C}.$	13.5	24.5	35.5
$\Delta, \text{equiv./ml./min.} \times 10^7$	3.88	1.96	0.90
$k' \text{ sec.}^{-1}$ for 1 mole trypsin per liter $\times 10^{-3}$	6.00	13.1	25.9
$k' (0^\circ) = 2.14 \times 10^3$	$\Delta H = 11,800$		

TABLE IV

Reaction mixture: 2 ml. of the substrate solution, 0.4 ml. 0.1 *M* phosphate buffer, pH 7.5; 0.4 ml. of beef chymotrypsin solution containing 0.105 mg. protein nitrogen per ml. in 7.5 phosphate buffer, diluted either 1/2 or 1/5.

$T, ^\circ\text{C}.$	35.7	24.7	13.3	4.0
$\frac{1}{2}$ period C.T. $\times 1/5$	8.7	17.5	35.0	—
$\frac{1}{2}$ period C.T. $\times 1/2$	—	6.5	[13.5]	25.0
			[12.5]	
$k (\times 1/5 \text{ min.}^{-1})$	0.080	0.041	0.021	0.011
$k, \text{sec.}^{-1}$ for 1 mole C.T. per liter $\times 10^{-2}$	3.5	1.8	0.93	0.49
$k (0^\circ) = 3.7 \times 10^2$	$\Delta H = 10,800$			

TABLE V

Reaction mixture: 4 ml. pepsin solution, 1 ml. chymotrypsin solution (0.021 mg. protein nitrogen per ml.). Taking molecular weight as 36,000, the molar concentration of chymotrypsin in reaction mixture is 7.5×10^{-6} mole per liter.

$T, ^\circ\text{C}.$	13.0	24.0	35
$\Delta, \text{equiv./ml./min.} \times 10^8$	3.75	7.85	16.2
$k' \text{ sec.}^{-1}$ for 1 mole C.T. per l. $\times 10^{-2}$	5.8	13.4	27.6
$k' (0^\circ) = 2.2 \times 10^2$	$\Delta H = 11,200$		

I have again to thank Dr. John H. Northrop for continued hospitality of his laboratory and for much valuable guidance. I am indebted to Dr. M. Kunitz for several of the substances used in this work.

SUMMARY

1. The velocity constants of the following reactions, (1) trypsin on benzoyl-*L*-arginine amide, (2) trypsin on sturin, (3) chymotrypsin on benzoyltyrosylglycylamide, (4) chymotrypsin on denatured pepsin, have been determined at various temperatures and the heats and entropy of activation have been calculated.

2. All these reactions come reasonably close to the range regarded as that of normal reactions among small molecules and, in fact, fairly close to the rates calculated by the simple collision hypothesis. The conversion of chymotrypsinogen by trypsin is considerably more rapid.

3. Since in highly specific reactions involving large molecules, it can hardly be expected that all collisions having the proper energy will lead to reaction, it is suggested that there is a compensating factor which produces an enhanced rate. This factor may arise from the formation of stable complex between enzyme and substrate.

INACTIVATION OF PEPSIN BY IODINE

II. ISOLATION OF CRYSTALLINE *L*-MONO-IODOTYROSINE FROM PARTIALLY IODINATED PEPSIN

BY ROGER M. HERRIOTT

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton,
New Jersey)

(Received for publication, May 12, 1941)

In a previous paper (Herriott, 1937) evidence was presented to show that iodine reacts with the tyrosine of pepsin and causes inactivation of the enzyme. After complete iodination and inactivation over 80 per cent of the iodine was recovered as di-iodotyrosine. Philpot and Small (1939) found that a small amount of iodine caused some inactivation of pepsin without any measurable drop in Folin's colorimetric tyrosine test and suggested, therefore, that some group other than tyrosine was involved. In the present work small amounts of iodine were introduced into the pepsin molecule and 65 per cent of this iodine was subsequently identified as *L*-mono-iodotyrosine. No evidence was obtained for any other iodine compound. The fact that mono-iodotyrosine has about 80 per cent of the molar color value of tyrosine instead of the 50 per cent of di-iodotyrosine explains the results of Philpot and Small.

The iodinated pepsin preparations were found to be fairly homogeneous as judged by a number of fractionation experiments. Crystallization of the iodinated pepsin and a subsequent solubility experiment showed the material to be very different from pepsin and to be relatively pure. The crystals were microscopically indistinguishable from those of pepsin. Electrophoresis failed to separate a mixture of pepsin and iodinated pepsin.

Iodination of Pepsin

Purified pepsin solutions (for preparation see Experimental Methods) were mixed with dilute alcoholic solutions of free iodine or dilute iodine in KI at pH 5.0-6.0 and room temperature until the iodine disappeared from solution. The iodine concentration had been adjusted so that there was a loss of only 10-20 per cent of the activity and no measurable change in the blue color value with Folin's phenol reagent. Half the free iodine that had disappeared from solution was found to be organically bound to the protein which is just what one would expect of a substitution reaction of iodine. Oxidation by iodine under these conditions is therefore improbable. This iodinated pepsin con-

tained about 0.7 per cent iodine or only 2 iodine atoms per molecule of pepsin. This amount is only one-twentieth of the amount of iodine previously found necessary to completely inactivate and completely iodinate pepsin. Iodide



FIG. 1 *a*. Crystalline *l*-mono-iodotyrosine from iodinated pepsin.
b. Crystalline di-iodotyrosine from iodinated pepsin.

ion was removed by dialysis or by washing the denatured protein precipitate on a funnel with $M/100$ sulfuric acid.

Crystallization of Iodinated Pepsin

Crystallization of iodinated pepsin was carried out in much the same way as for the original pepsin, *i.e.* precipitation by titrating to pH 2.5; stirring the filter cake in one-half its volume of water at 35° C. plus just enough $N/2$ sodium hydroxide to dissolve the solid, then stirring and cooling slowly.

The resulting crystals were indistinguishable under the microscope from ordinary pepsin crystals. Only one preparation was crystallized. No other attempts were made since there was no indication of any change in properties by crystallization.

Tests of Purity

It was thought important to determine whether the iodinated pepsin preparations were reaction mixtures of several proteins of varying iodine content or if the material was fairly homogeneous. This would have some bearing on the question as to the amount of iodine necessary to inactivate a pepsin molecule.

TABLE I
Fractionation Experiments

Sample No	Materials and Procedure	$\frac{\text{Hb}}{\text{mg. P. N.}}$ [P.U.]	Iodine per cent
1	Original pepsin	0.32	
2	No. 1 after iodination*	0.23	1.4
3	A fraction of No. 2 insoluble in 0.5 sat. MgSO_4 0.05 M pH 4.6 acetate buffer	0.22	
4	First crystals of No. 2	0.23	1.4
5	Mother liquor from first crystals	0.22	1.3
6	Fraction of No. 4 soluble in 0.25 M Na_2SO_4 pH 4.0	0.21	1.45
7	Fraction of No. 4 that did not dissolve in aliquot of 0.25 M Na_2SO_4 —pH 4.0 used in No. 6	0.21	1.0

* More iodine was added to this particular preparation than to the others which accounts for its higher iodine content and lower specific activity $\left[\frac{\text{Hb}}{\text{mg. P. N.}}\right]$.

Iodinated pepsin preparations were therefore subjected to various fractionating tests and the various fractions analyzed.

It is apparent from the results in Table I and Fig. 3 that the preparations are relatively homogeneous and do not consist of an inactive iodinated protein mixed with some of the original pepsin. It is likely then that the iodine distributes itself fairly evenly among all the protein molecules.

Electrophoresis.—Electrophoresis of a solution of the crystalline iodinated pepsin in the Tiselius cell (kindly analyzed by Dr. Alexandre Rothen) at pH 4.4 revealed that there was only one moving boundary. However, when this same material was mixed with equal amounts of pure pepsin the mixture also showed only a single sharp boundary both before reversing the current and afterwards (Fig. 2). This was done at only one pH and it is possible that at some other pH they could be separated. The mobilities were as follows: for the ascending

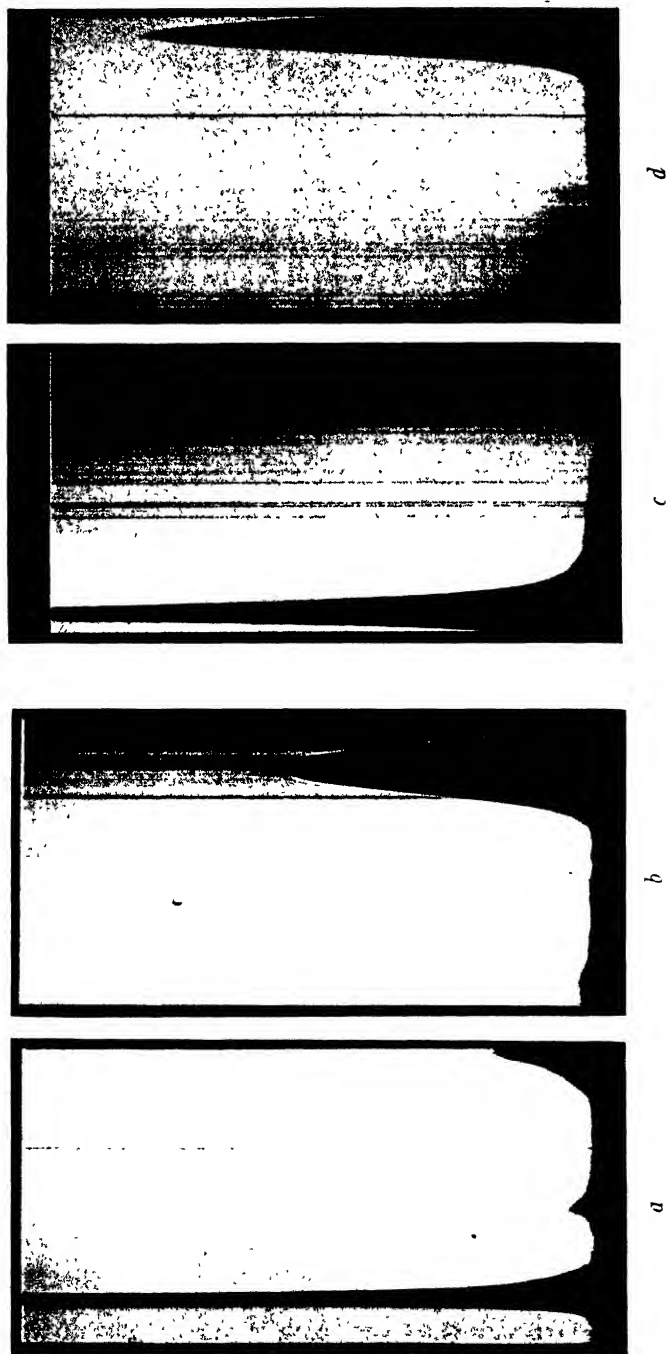


FIG. 2. Electrophoresis patterns of an artificial mixture of pepsin and iodinated pepsin at pH 4.4; specific conductivity 0.00069 reciprocal ohms. *a* and *b* are the ascending and descending boundaries after 2460 seconds; *c* and *d* are the ascending and descending boundaries after reversing the direction of the current and running for 2640 seconds.

boundary of the iodinated pepsin 8.1×10^{-5} and the descending boundary 7.8×10^{-5} . In the mixture of iodinated pepsin and pepsin the ascending boundary was 7.8×10^{-5} while the descending boundary was 7.5×10^{-5} , all values being expressed in $\text{cm}^2/\text{volt}/\text{sec}$.

Solubility Curve.—A solubility curve of the crystalline pepsin is shown in Fig. 3. In the solvent used, 0.25 M sodium sulfate pH 4.0, crystalline pepsin is at least 20 times as soluble as the crystals of iodinated pepsin. The two proteins can therefore easily be distinguished. The curve in Fig. 3 is not that of an ideal substance but it shows no great degree of inhomogeneity. The points marked \circ and \square are values determined and calculated when some pepsin was added to the solvent before adding the crystalline iodinated pepsin. The value \square was expected only if the solubilities of the two proteins were independent of each other; *i.e.*, they form a mixture. Since the value fell considerably below

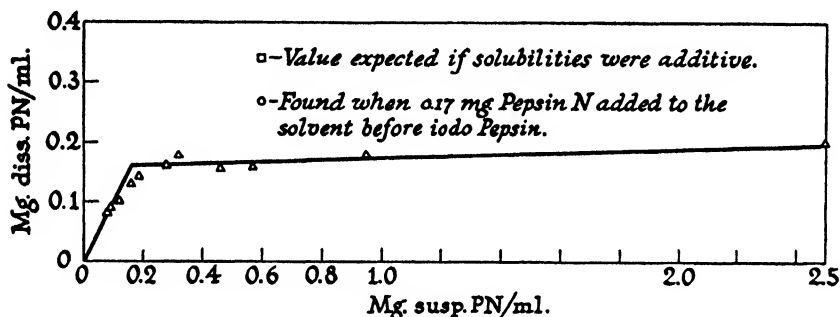


FIG. 3. Solubility of crystals of iodinated pepsin in 0.25 M Na_2SO_4 pH 4.0 at 23°C.

the expected, it may be concluded that the iodinated pepsin forms solid solutions with ordinary pepsin.

The solubility of iodinated pepsin will probably decrease as more iodine is introduced into the molecule.

Isolation of l-Mono-Iodotyrosine from Iodinated Pepsin

The experimental procedure for the isolation of pepsin and subsequent isolation of *l*-mono-iodotyrosine is shown in Table II. Fig. 1 *a* shows the appearance of the crystalline *l*-mono-iodotyrosine. In one preparation a small amount of di-iodotyrosine was also crystallized out (Fig. 1 *b*).

As may be seen in Table II, solution No. 13, contains 65 per cent of the original iodine. This was successively extracted with butyl alcohol until over 80 per cent of the total iodine had been extracted. The fraction extracted by each aliquot of butyl alcohol was reasonably constant over the whole range as may be seen in Fig. 4. Under similar conditions the fraction of di-iodotyrosine extracted is 50 per cent instead of the 20 per cent, as shown in Fig. 4. It could

TABLE II
Isolation of L-Mono-Tyrosine from Iodinated Pepsin

Materials and procedures	No.	Vol.	Total N	Total I	Phenol ^o I	I [†] N	Phenol [‡] N
		ml.	mg.	mg.			
Dialyzed purified pepsin solution, pH 5.3	1	1075	5700				
No. 1 + (20 ml. 4 M NaAc to pH 5.7 + 5.5 ml. N/1 alcoholic iodine) slowly at 15°C. Allowed to stand until colorless. The specific activity had dropped 11 per cent.	2	1100					
No. 2 + 5 ml. 5 N NaOH to pH 7.0, warmed to 60°C. for 5 min. then + 50 ml. 5 N H ₂ SO ₄ and filtered and washed twice with 100 ml. of M/100 H ₂ SO ₄ . Residue dissolved in NaOH + H ₂ O....	3	500	5650	300			
No. 3 + 200 gm. Ba(OH) ₂ crystals and refluxed 20 hrs., then cooled and left 50 hrs. at 5°C. Filtered and residue washed. Filtrate.	4	650		310			
No. 4 heated to 90°C. then + 115 ml. 5 N H ₂ SO ₄ with stirring to pH 3.5 and aerated for 1 hr. to remove H ₂ S. 200 ml. M/1 PbAc ₂ added and solution filtered and residue washed three times with 80 ml. each of N/10 acetic acid. Filtrate and washings.	5	1300		307			
No. 5 + 85 ml. 5 N NaOH pH 8.5; let stand, filter, and wash on funnel. Filtrate and washings.	6F	1400		56			
Precipitate.	6P						
No. 6P stirred with dilute acetic acid to pH 4.0, then + HCl to pH 2.0, filter. Filtrate diluted to 1 liter; precipitate appeared which was filtered off and discarded. Filtrate (pH 4.3). Titrated to pH 8.0 and filtered. Filtrate.	7F	1000		16			
Precipitate.	7P						
No. 7P + dilute acetic acid to pH 4.0. Filtered. Filtrate.	8F						
Precipitate.	8P						
No. 8P + HCl to dissolve, then + NaAc to pH 4.0; filter. Filtrate.	9F	50	4.5	24	1.15	5.3	6.2
No. 8F + alkali to pH 8.0, filter and wash residue. Filtrate + washings.	10F	500		10			
Precipitate.	10P						
No. 10P + H ₂ SO ₄ to pH 3.0, filtered and washed with H ₂ O. Filtrate + washings.	11F	85	23.5	150	1.4	6.5	9.1
Precipitate.	11P						

TABLE II—Continued

Materials and procedures	No.	Vol.	Total N	Total I	Phenol* I	I† N	Phenol‡ N
		ml.	mg.	mg.			
No. 11P ground in mortar with N/20 H ₂ SO ₄ , filtered. Filtrate.....	12	42	7.1	29	1.6	4.0	6.2
No. 9F + No. 11F + No. 12 con- centrated <i>in vacuo</i>	13	84	33.5	193	1.5	5.75	8.8
No. 13 diluted to 100 ml. with dilute H ₂ SO ₄ , pH 2.5 then + 50 ml. butyl alcohol saturated with water and shaken 3 min. in separatory funnel. Separate. Butyl alcohol layer.....	14A	38.5		49	1.6	6.6	10.5
Water layer.....	14W	111.0		145			
No. 14W + 38 ml. butyl alcohol satu- rated with H ₂ O, shaken, and separated.	15A	38		39			
	15W	111		106			
No. 15W + 38 ml. butyl alcohol satu- rated with H ₂ O, shaken, and separated.	16A	38		23			
	16W	110		83			
No. 16W + 38 ml. butyl alcohol satu- rated with H ₂ O, shaken, and separated.	17A	38		17.5	1.2	7.4	8.9
	17W	108		66			
No. 17W + 38 ml. butyl alcohol satu- rated with H ₂ O, shaken, and separated.	18A	38		13.7			
	18W	104		52			
No. 18W + 38 ml. butyl alcohol satu- rated with H ₂ O, shaken, and separated.	19A	39		10			
	19W	103		43			
No. 19W + 38 ml. butyl alcohol satu- rated with H ₂ O, shaken, and separated.	20A	39					
	20W	102					
No. 20W + 38 ml. butyl alcohol satu- rated with H ₂ O, shaken, and separated.	21A	39		6.8	1.5	6.8	10.0
	21W	101		28.6	2.7	2.1	5.7
All of the butyl alcohol solutions from 14A–21A collected and evaporated <i>in vacuo</i> to 50 ml. This was then ex- tracted with 25 ml. saturated Ba(OH) ₂ solution + 25 ml. water. Alkali water layer.....	22	48		120			
No. 22 + H ₂ SO ₄ to pH 3.0, filter and wash residue; evaporate to 10 ml. then + 5 N NaOH to pH 3.5, let stand at 5°C.; a small brown precipitate came out, was centrifuged off, and dis- carded. Titrate supernatant to pH 5.7 and let stand. Rosettes of diamond-shaped platelets appeared. Place in shallow dish and evaporate to 2.3 ml., cool, filter. Filtrate + washings.....	23F	1.5		22.5			
Crystalline precipitate.....	23P						

TABLE II—*Concluded*

Materials and procedures	No.	Vol.	Total N	Total I	Phenol* I	I† N	Phenol‡ N
		ml.	mg.	mg.			
No. 23P dissolved.....	24	12.5	9	88	1.0	10.0	9.6
No. 21W + various residues + washings.....	25	22		57			
No. 25 extracted twice with 50 ml. butyl alcohol.....	26A 26W	115 15		55 2			
No. 26A extracted with Ba(OH) ₂ ; acidify water layer with H ₂ SO ₄ , centrifuge; supernatant + 23F evaporated down. Diamond platelets again formed; Filter and wash residue once.							
Filtrate.....	27F	5.5		25	2.7	2.8	7.6
Crystals dissolved.....	28P	9.3		37	1.8	6	10.8
No. 24 + No. 28P.....	29			125			
No. 29 recrystallized.							
Crystals dissolved.....	30P	9	10	100	1.13	9	10
Mother liquor.....	30F	6.5	8	30	1.9	4.1	7.7

* This ratio is number of milligrams of tyrosine that give the equivalent color with Folin's phenol reagent divided by the number of milligrams of iodine.

† This ratio is the number of milligrams of iodine of a sample divided by the number of milligrams of nitrogen.

‡ This ratio is the same equivalent color as in the first footnote (*) but divided by the number of milligrams of nitrogen.

be concluded from this distribution experiment, therefore, that the iodine-containing component was probably not di-iodotyrosine and that the component was fairly homogeneous with respect to iodine; *i.e.*, there was only one iodine component. It should be pointed out that the analyses of the fraction in the first butyl alcohol extract, 14A of Table II, are practically the same as the analyses of the last extract, 21A. This is additional evidence regarding the homogeneity of this material. In Fig. 4 the ordinate scale spacing is logarithmic.

Although only 42 per cent of the original iodine was obtained as crystalline mono-iodotyrosine, the distribution or extraction experiment makes it highly probable that over 65 per cent of the original iodine was present as iodinated tyrosine.

The details of the distribution experiment are given in Table II but a few comments are appropriate. Butyl alcohol and water have an appreciable solubility in each other. Therefore for constancy of volume it is necessary to saturate each with the other. In Table II, No. 14 of the water solution was not initially saturated with butyl alcohol but after the first extraction with 50 ml. of butyl alcohol the butyl

alcohol volume decreased to 38 ml. and the difference was the volume of butyl alcohol necessary to saturate the water layer. To simplify the problem, therefore, in the second and succeeding extractions 38 ml. of butyl alcohol saturated with water was used. By doing this the volumes remained constant.

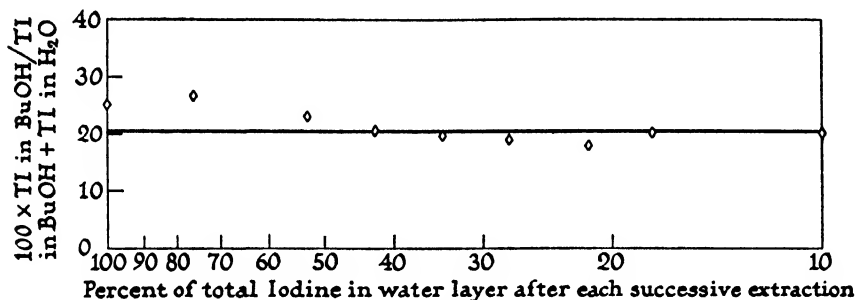


FIG. 4. Distribution of iodine components between butyl alcohol and water at pH 3.0 and 25°C. on successive extractions.

Identification and Properties of l-Mono-Iodotyrosine

Mono-iodotyrosine was isolated only recently by Ludwig and Mutzenbecher (1939) from iodinated casein. They measured very few properties but state that, as compared to di-iodotyrosine the mono- derivative had the same crystalline appearance, was more soluble in water, had the same melting point, and, of course, differed primarily in its iodine content.

Table III shows the results we have obtained compared to those of di-iodotyrosine. On one occasion the mono- derivative crystallized out with rounded edges appearing somewhat like the whetstones of di-iodotyrosine but most of the time they appeared as sharply formed diamond platelets as shown in Fig. 1 *a* or rosettes of these platelets.

The iodine and nitrogen values obtained are slightly different from those reported by Ludwig and Mutzenbecher but they found one molecule of water of crystallization in their product. Since our product was dried at 70°C. *in vacuo* this water was presumably driven off, thus accounting for the discrepancy. Our values agree quite closely to the theoretical percentages of an anhydrous material.

In view of the fact that Ludwig and Mutzenbecher had also obtained thyroxine from iodinated casein it was thought that possibly our crystalline iodine derivative might be related to thyroxine perhaps as an ether of one molecule of ordinary tyrosine and one of di-iodotyrosine. Such a compound would yield elementary analyses close to those of mono-iodotyrosine. However, a molecular weight determination by the Barger vapor pressure method (1904) using formic acid as the solvent and di-iodotyrosine as the standard showed the

compound to have a molecular weight in the neighborhood of 300 which rules out the ether possibility.

The monoiodo derivative gives a strong Millon's reaction so that one should be careful of concluding that tyrosine has not been iodinated merely from a positive Millon reaction even though carried out quantitatively.

TABLE III
Properties of Crystalline l-Mono-Iodotyrosine Compared to Di-Iodotyrosine

Property	Monoiodo-	Diiodo-
Crystalline form.....	diamond platelets	whetstones or needles
[α] _D in 4 per cent HCl at 22°C.....	-8.8	-2.9*
Solubility in water in mg./ml.....	4	0.8
Melting or decomposition point.....	201°C. corrected	195°C. corrected
Phenol color value/iodine, ratio†.....	1.1	0.3
Phenol color value/nitrogen, ratio†.....	10.0	6.2
Iodine/nitrogen, ratio†.....	9.0	18.0
Reaction to Millon's reagent.....	+	-
Nitrous acid color test for ortho-iodo-benzene derivatives‡.....	+	+
Per cent iodine.....	42.0 found§ 41.5 calculated	
Per cent nitrogen.....	4.5 found§ 4.55 calculated	

* Value from Abderhalden (1923)

† These ratios are explained in the footnotes of Table II

‡ Kendall and Osterberg (1919)

§ The sample used for these analyses was dried 15 hours at 70°C. *in vacuo*.

EXPERIMENTAL METHODS

The extraction procedure consists of agitation by hand in a separatory funnel for 3 minutes at room temperature after which the solutions are allowed to separate and each layer drawn off and analyzed for total iodine. The pH of the water solution was adjusted at the start to between pH 2.3 and pH 3.3 with sulfuric acid.

Preparation of Pepsin.—The pepsin used through out this work was prepared according to Table VI of a previous publication (Herriott, Desreux, and Northrop, 1940). The protein in the filtrate No. 4 of that table was precipitated by addition of 250 gm. of solid $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ for every liter of filtrate. The precipitate was filtered off and dialyzed in cellophane bags against tap water overnight.

Determination of Nitrogen.—This estimation was described by Northrop and Kunitz (1932). Instead of distilling into hydrochloric acid as previously done, a 4 per cent boric acid solution with brom cresol green indicator is used. Only one titration is required and this is with N/70 hydrochloric acid. This change was brought to the writer's attention by Dr. Bacon F. Chow.

Determination of Iodine.—A modification of the method of Kendall, as previously described by Herriott (1937) was used.

Determination of Phenol Color.—1 ml. of a solution of material which would yield about the same intensity of blue color as 0.15 mg. of tyrosine was put into a 50 ml. Erlenmeyer flask; 8 ml. of 0.5 N sodium hydroxide was added, followed by 3 ml. of a 1/3 dilution of Folin's phenol reagent prepared according to Folin and Ciocalteu (1927). The phenol reagent was added dropwise with stirring and the color read after 10 minutes against a tyrosine standard treated in the same way or a calibrated blue glass. When the unknown contains a high concentration of buffering material more alkali is required.

Peptic Activity Measurements.—The hemoglobin activity method was that of Anson (1938). The rennet activity method was that of Herriott (1938).

Solubility Methods.—This has been discussed in detail by Herriott, Desreux, and Northrop (1940).

SUMMARY

1. Pepsin solutions were iodinated at pH 5.0–6.0 until 10–20 per cent of the activity was lost and 1/20 (0.7 per cent) of the saturating amount of iodine had been introduced into the protein molecule. After alkaline hydrolysis 65 per cent of the original iodine was accounted for as mono-iodotyrosine although only 42 per cent was isolated as a crystalline product. No evidence was obtained to support the possibility that any group other than tyrosine in pepsin was iodinated.

2. Some of the properties of the crystalline *l*-mono-iodotyrosine were determined and compared to those of di-iodotyrosine.

3. One iodinated pepsin preparation was crystallized. The crystal form was the same as that of the original pepsin. A solubility curve of the crystals demonstrated that it was very different from pepsin and had nearly constant solubility.

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BACTERICIDAL SUBSTANCES FROM STERILE CULTURE-MEDIA AND BACTERIAL CULTURES

WITH SPECIAL REFERENCE TO THE BACTERIOLYTIC PROPERTIES
OF ACTINOMYCETES

By MAURICE WELSCH¹

(From the Laboratories of The Rockefeller Institute for Medical Research,
Princeton, New Jersey)

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INTRODUCTORY

Lysis of susceptible bacteria suspended in water or agar by different microorganisms has been frequently reported in the literature (Nicolle, 1907; Schiller, 1914-1933; Ido, 1931; Callerio, 1932; Callerio and Dolcini, 1932; Bernard *et al.*, 1937).

The particular case of bacteriolysis by actinomycetes has been described independently by Lieske (1921), and by Gratia and Dath (1924-1931). The latter obtained antigenic, sterile, lysates and used them for therapeutic purposes (Gratia, 1930, 1931, 1934).

Working with an *Actinomyces* of the *albus* group, isolated from the air of the laboratory,² the writer has shown that, to obtain constant lysis, it is necessary to introduce into the susceptible suspension of dead or living bacteria some medium from a sporulated (Welsch, 1937 b, c, e, 1938d) culture of the *Actinomyces* sp. together with this organism (Welsch, 1936, 1937f).

For the sake of convenience, the sterile filtrate (or supernatant after centrifugalization) from a sporulated broth-culture of our *Actinomyces* sp. has been designated as "actinomycetin" (Welsch,

¹ Fellow of the Belgian American Educational Foundation, Inc. Now with the Department of Soil Microbiology, Rutgers University, New Brunswick, N. J.

² This organism has not been fully identified and will be designated as *Actinomyces* sp. Other strains have been isolated which possess the same general properties but, so far, have not been so extensively studied.

1937c) and will be so referred to in this paper. It is one of the purposes of this contribution to show that crude actinomycetin contains at least two different substances acting on certain bacteria: a bacteriolytic protein which dissolves dead bacterial cells and a bactericidal ether-soluble agent which kills especially the gram-positive organisms.

EXPERIMENTAL

Gram-negative bacteria, killed by heat or by chemicals, are readily dissolved by diluted actinomycetin. Evidence has been submitted to prove that the lytic agent is a protein (Welsch, 1937d, 1938c, 1939b; Welsch and Elford, 1937), and an enzyme (Welsch, 1938a, b, 1939a). Killed gram-positive bacteria, though susceptible to some extent, are more resistant.

A few strains of streptococci and pneumococci are susceptible to sterile actinomycetin even when alive. Many other living gram-positive organisms, though resistant to sterile actinomycetin, are dissolved after two or three days when their aqueous suspensions are inoculated with a suitable *Actinomyces* and some actinomycetin (Welsch, 1937a). Living gram-negative bacteria are very resistant to both actinomycetin and *Actinomyces* sp.

It was thought that the gram-positive organisms which may be dissolved by the growing *Actinomyces* sp. but are unsusceptible to sterile actinomycetin might be lysed by concentrated and purified preparations of the bacteriolytic protein (Welsch, 1937a). Accordingly, crude actinomycetin was brought to 0.75 saturation by addition of solid $(\text{NH}_4)_2\text{SO}_4$; the precipitate was recovered after filtration through filter-cel highflow and dissolved in a small amount of distilled water. This procedure was repeated three times in succession; the last precipitate titrated 100 times more mycolytic units per milliliter than crude actinomycetin and 10 times more activity per milligram of protein. Those preparations, powerful when tested on heat-killed bacteria, do not clarify the suspensions of living cells. It seems, therefore, that the protein responsible for the lysis of dead bacteria is only able to play, at most, a secondary rôle in the dissolution of living organisms by our *Actinomyces*.

The presence of a bactericidal agent in actinomycetin was then investigated. Though crude actinomycetin has no appreciable bactericidal action, it was found that the concentrated preparations, obtained by the method outlined above, are slightly bacterio-toxic. When 1 ml. of such a concentrated preparation is added to 10 ml. of nutrient agar which is then plated, the growth of several gram-positive bacteria on this medium is inhibited, provided that a small inoculum is used. The bactericidal agent was extracted and titrated as follows.

Extraction of the bactericidal substances. Actinomycetin, (and also other bacterial cultures or sterile media) are brought to 0.75 saturation by addition of solid $(\text{NH}_4)_2\text{SO}_4$ or titrated to pH 3 or 4 with HCl; the precipitate is recovered by filtration through filter-cel; the filter-cake is dried at low temperature, powdered and extracted several times with sulfuric ether. The ether extract is, at last, concentrated by distillation under reduced pressure.

Test for bactericidal activity. Suitable dilutions of the ether extract are made up in 1/50 Mol. phosphate buffer pH 7.4 in 0.85 per cent NaCl. A standard culture of non-sporulating *Bacillus megatherium* (36 S) is prepared according to Northrop, 1939. This culture, which contains very regularly 10^7 cells per milliliter, is diluted to 1/10 in phosphate buffer. Then, 0.5 ml. of this dilution is added to 0.5 ml. of each dilution of the extract to be tested. The suspensions are next incubated for 2 hours at 37° ; then, they are diluted to 1/500 and 1 ml. of each is plated for colony count according to the technique described by Gratia (1936) for enumeration of bacteriophage plaques.

Using the above methods, it was found that not only actinomycetin but also several sterile culture-media yielded some bactericidal activity, though to a lesser extent. To compare the activity of extracts from different origins, it was found convenient to express the amount of active material tested in term of a "standard dilution."

Standard dilution. This is a dilution of the ether extract which contains in 0.5 ml. the amount of active material extracted from 1 ml. of the original bacterial culture or sterile medium. Thus, when 0.5 ml. of this standard dilution is tested in 0.5 ml. of the

susceptible bacterial suspension, the concentration of the active agent corresponds to the amount originally contained in 1 ml. of the initial, crude material. The activity of the standard dilution is then, so to speak, a measure of the "latent toxicity" of the crude material studied.

The dry weight of ether-soluble material extracted from 1 liter of bacterial culture or sterile medium is given in the tables for each particular case. By definition, when the standard dilution is tested, 1/1000 of this quantity acts upon 0.5 times 10^6 susceptible bacteria suspended in 1 ml. of buffer.

TABLE 1

Extraction of bactericidal agents from actinomycetin and from sterile culture-media
(Precipitation by $(\text{NH}_4)_2\text{SO}_4$)

DILUTION OF EX-TRACT TESTED	FROM 1 LITER OF:			
	Broth	1 per cent Fairchild peptone	Yeast extract	Actinomycetin (broth)
	Milligrams extracted			
	27.2	37.7	12.8	42.3
	Number of colonies actually observed after action of bactericidal extract			
"Standard"	0	0	56	0
1/2	0	15	1018	0
1/4	76	1068	1046	0
1/8	973	996		56
1/16	1024	1049		659

Control: 1056.

It must be pointed out, however, that the measurement of bactericidal activity on a dry-weight basis has no special significance since the ether-extracts tested are different mixtures of active and inactive substances in variable and unknown proportions.

The results obtained with ordinary broth, 1 per cent Fairchild peptone, yeast-extract (Northrop, 1939) and actinomycetin are shown in table 1.

Bactericidal substances wholly produced by *Actinomyces* sp. were obtained in the following way. Ordinary broth was titrated to pH 3 with HCl; the precipitate was removed by filtration

through filter-cel, dried, extracted with ether and the activity of the extract tested. The filtrate was titrated to pH 7.6 with normal NaOH, sterilized and inoculated with *Actinomyces* sp. After incubation for 5 days at 37°, the actinomycetin thus obtained was acidified to pH 3 with HCl, the precipitate was recovered after filtration through filter-cel, dried and extracted; then, the activity of the extract was measured. The results of this experiment are shown in table 2.

Active material of bacterial origin, only slightly contaminated by bactericidal substances from the medium, may be obtained

TABLE 2

Separation of bactericidal substances from actinomycetin and from culture-media
(Precipitation by HCl)

DILUTION OF EXTRACT TESTED	FROM 1 LITER OF:		
	Broth	Actinomycetin (broth)	Actinomycetin (broth + HCl)
	Milligrams extracted		
	22.6	39.5	41.6
	Number of colonies actually observed after action of bactericidal extract		
"Standard"	0	0	0
1/2	0	0	0
1/4	676	86	0
1/8	1106	754	123
1/16	1097	1059	892

Control: 1088.

after culture in yeast-extract which contains less of those agents than the ordinary complex media.

Bactericidal activity was obtained from cultures in 1 per cent Fairchild peptone or in yeast-extract of *Bacillus subtilis*, *Bacillus mycoides*, *Bacillus mesentericus* and *Pseudomonas aeruginosa* isolated from the air of the laboratory. A collection strain of non-sporulating *Bacillus megatherium* (36 S) failed to give appreciable amounts of bactericidins. Those results are shown in table 3.

The general properties of the active material from the different cultures and sterile media appeared to be alike in all cases. It is soluble in ether, petroleum ether, benzen, carbon tetrachloride,

acetone, chloroform; less soluble in ethyl alcohol; insoluble in water and dilute acids but soluble in dilute sodium hydroxide. It is thermostable.

The bactericidal activity is best demonstrated with suspensions of gram-positive organisms in buffer (including the vegetative cells of those spore-bearing bacteria which produce the bactericidal agent). Larger amounts of material prevent the growth of gram-positive bacteria in culture media, better results being obtained with solid media. Gram-negative organisms, as a rule

TABLE 3
Formation of bactericidal substances by various bacteria
(Cultures in 1 per cent peptone, precipitation by HCl)

DILUTION OF EXTRACT TESTED	FROM 1 LITER OF:					
	1 per cent peptone	<i>B.</i> <i>megatherium</i>	<i>B.</i> <i>subtilis</i>	<i>B.</i> <i>mycoides</i>	<i>B.</i> <i>mesentericus</i>	<i>P.</i> <i>aeruginosa</i>
	Milligrams extracted					
	14.2	16.7	33.8	36.2	34.6	40.8
	Number of colonies actually observed after action of bactericidal extract					
"Standard"	58	6	0	0	0	0
1/2	647	92	0	0	0	0
1/4	1108	1087	87	19	2	0
1/10	1024	1045	894	953	247	114

Control: 1065.

are much more resistant. Table 4 shows some of the results obtained with ether extract from actinomycetin. The number of susceptible cells inoculated influences greatly the results as shown in table 5.

The toxic action of the material studied is not limited to bacterial species only. The growth of tomato roots in pure culture (White, 1934, 1939) was found to be inhibited by the highest dilutions active upon *B. megatherium* in buffer.³ Those dilutions induce an immediate cytolysis of several ciliates in water (*Paramoecium*, *Glaucoma*, *Colpidium*); even higher dilutions prevent the multiplication of those protozoa in pure culture (Glaser and

³ The writer is indebted to Dr. P. R. White for those experiments.

TABLE 4

Action of bactericidal agent from actinomycetin on the growth of various bacteria in liquid culture-media*

0.01 ML. FROM A 24-HOUR-OLD CULTURE OF THE FOLLOWING SPECIES INOCULATED IN 2 ML. FRESH MEDIUM + BACTERICIDAL EXTRACT		CULTURE MEDIUM	AMOUNT OF BACTERICIDAL AGENT, MG./ML.						
			0.8	0.4	0.2	0.1	0.05	0.025	0.00
Gram-negative									
<i>Pseudomonas aeruginosa</i>	a	++	++	++	++	++	++	++	++
<i>Phytomonas phaseoli</i>	c	++	++	++	++	++	++	++	++
<i>Phytomonas ricinicola</i>	c	++	++	++	++	++	++	++	++
<i>Escherichia coli</i>	a	++	++	++	++	++	++	++	++
<i>Erwinia tracheiphila</i>	c	++	++	++	++	++	++	++	++
<i>Serratia marcescens</i>	c	++	++	++	++	++	++	++	++
<i>Salmonella schottmuelleri</i>	a	++	++	++	++	++	++	++	++
<i>Salmonella abortusovae</i>	a	++	++	++	++	++	++	++	++
<i>Salmonella choleraesuis</i>	a	++	++	++	++	++	++	++	++
<i>Pasteurella pseudotuberculosis</i>	b	++	++	++	++	++	++	++	++
<i>Brucella abortus</i>	b	++	++	++	++	++	++	++	++
<i>Hemophilus influenzae</i>	d	+	++	++	++	++	++	++	++
<i>Hemophilus suis</i>	d	+	+	++	++	++	++	++	++
<i>Flavobacterium</i> sp.....	a	0	0	0	+	++	++	++	++
Gram-positive									
<i>Staphylococcus muscae</i>	b	++	++	++	++	++	++	++	++
<i>Corynebacterium pyogenes</i>	d	+	++	++	++	++	++	++	++
<i>Erysipelothrix rhusiopathiae</i>	d	+	++	++	++	++	++	++	++
<i>Phytomonas flaccumfaciens</i>	c	+	++	++	++	++	++	++	++
<i>Staphylococcus aureus</i>	b	0	0	0	+	++	++	++	++
<i>Bacillus mesentericus</i>	a	0	0	0	+	++	++	++	++
<i>Bacillus subtilis</i>	a	0	0	0	0	+	++	++	++
<i>Streptococcus pyogenes</i>	d	0	0	0	0	+	++	++	++
<i>Diplococcus pneumoniae</i>	d	0	0	0	0	+	++	++	++
<i>Mycobacterium phlei</i>	b	0	0	0	0	+	++	++	++
<i>Bacillus mycoides</i>	a	0	0	0	0	0	+	++	++
<i>Bacillus megatherium</i> (36 S).....	a	0	0	0	0	0	+	++	++

a = yeast extract; b = broth; c = broth + 1 per cent glucose; d = broth + 1 per cent horse-blood.

++ = normal growth; + = growth poor or delayed; 0 = no growth (microscopical examination daily for 6 days).

* The writer is indebted to Drs. C. TenBroeck, J. H. Northrop, R. E. Shope and K. W. Kreitlow for many of the strains used for this experiment.

Coria, 1930-1933).⁴ This should be compared to the findings of E. and M. Chatton (1927).

⁴ The writer is indebted to Dr. R. W. Glaser for the pure cultures of protozoa tested.

On account of technical difficulties in the preparation of actinomycin on a large scale, no attempt was made to purify the bactericidal agent from *Actinomyces* sp. No further investigations were made with broth or peptone, since it was found difficult to obtain quantitatively reproducible results with successive batches of these media. On the other hand, investigations are under way to isolate the active material from *B. subtilis* grown in yeast extract and from dry yeast. In both cases, the fractionation of the ether extract has given three different portions; the first one, obtained by extracting the ether solution with dilute HCl, has no bactericidal activity; the second one, obtained by extraction of

TABLE 5

Influence of the size of inoculum on the action of the bactericidal agent from actinomycin

NUMBER OF CELLS (STAPHYLOCOCCUS AU- REUS) INOCULATED IN 1 ML. BROTH + 0.05 MG. ETHER-SOLUBLE AGENT	GROWTH OF THE CULTURE AFTER INCUBATION AT 37° FOR HOURS			
	24	48	72	96
10 ⁶	+	+	+	+
10 ⁵	0	+	+	+
10 ⁴	0	+	+	+
10 ³	0	0	+	+
10 ²	0	0	0	0
10	0	0	0	0

Controls without ether-extract grow in 24 hours.

the ether solution with dilute NaOH, contains most of the activity. The third one, which cannot be extracted from the ether either by dilute acid, or by dilute alkali, shows a slight bactericidal activity. This last portion, after hydrolysis by boiling concentrated alkali, may in turn be fractionated in three portions; the one soluble in dilute alkali is the only one which exhibits some activity. The best preparations so far obtained are definitely acidic; they appear as a yellowish, oily material which kills *B. megatherium*, under the conditions of our test, in concentration of 5 micrograms per milliliter.

DISCUSSION

From several ordinary sterile culture-media, an ether-soluble bactericidal substance has been extracted, which readily kills the

susceptible bacteria in buffer at a dilution corresponding to its actual concentration in the medium. Since the susceptible bacteria are able to grow very well in such media, it is obvious that, before extraction, the bactericidal agent must exist in a harmless form. Its harmlessness may be due, for instance, to its association with some other molecule; its precipitability by $(\text{NH}_4)_2\text{SO}_4$ suggests that it might be associated with some protein. The fact that, after extraction, the active substance kills readily the susceptible organisms suspended in buffer but is very much less potent in culture-media, indicates also that, in complex media, the bactericidin is, at least partly, transformed into a harmless substance.

When several of the organisms tested have been grown in a given medium, the amount of activity which may be extracted from such a culture is definitely greater than what may be obtained from the sterile medium. This indicates that, undoubtedly, part of the active material has a bacterial origin. Crude sterile filtrates from the cultures of the organisms studied have no appreciable bactericidal action; their ether-extracts, while acting well in buffer, are much less potent in culture-media. Again, this suggests that the active substance, in complex culture-media, is, in some way, inhibited.

The particular case of *Actinomyces* sp., which we have more extensively studied, may be summarized as follows. Crude actinomycetin has no appreciable bactericidal activity and is unable to dissolve living cells (with a few exceptions); but, the living *Actinomyces* itself, growing in a suspension of susceptible bacteria in buffer, brings about the lysis of the cells. On the other hand, we have shown that actinomycetin contains an enzyme which dissolves dead bacteria and sufficient amounts of a bactericidal agent which, however, is in a harmless form. It seems therefore reasonable to assume that the rôle of the *Actinomyces* in the phenomenon of bacteriolysis is either to secrete new bactericidal substances in the active form or to remove the unknown factor which inhibits the action of the bactericidins already present in actinomycetin; for instance, it might free the bactericidal agent from its hypothetical harmless complex. This last mechanism seems the more likely since constant lysis of living bacteria is obtained

only when some actinomycetin is added with the *Actinomyces* sp. to the susceptible suspension.

The mechanism here suggested to explain the facts observed with our *Actinomyces* sp. is not supposed to cover all the antagonistic activities of other actinomycetes. It should be emphasized, on the contrary, that other species exhibit antagonistic properties of a different kind, as indicated by the works of Greig-Smith (1917), Tims (1932), Borodulina (1935), Nakhimovskaia (1937), Waksman and Foster (1937), Krassilnikov and Koreniako (1939), Waksman and Woodruff (1940), and Kriss (1940).

In a personal communication to Dr. Gratia, Dr. Schiller suggested that Gratia and Dath's observation of the bacteriolytic activity of some actinomycetes was a particular case of his "induced" antagonism. It may be recalled here that, according to Schiller (1914-1933), when two suitable bacterial species are incubated together in some fluid or agar in which an essential nutrient, such as nitrogen for instance, is lacking, one of the two species is able to digest the other, through the agency of more or less specific lysins newly formed by a process of adaptation; hence the name of "induced" antagonism. We have never observed the production of new adaptive lysins or bactericidins by *Actinomyces* sp. grown in association with susceptible bacteria in buffer, water or water-agar. On the contrary, the amount of lytic enzyme or of ether-soluble bactericidal agent obtained after culture of *Actinomyces* sp. in a susceptible bacterial suspension, is definitely smaller than after culture in an artificial medium. The active substances obtained from mixed cultures never show any increase of specificity. The antagonistic relationship studied by us is then entirely different from Schiller's induced antagonism.

On the other hand, our observations on the action of several proteolytic bacteria on other organisms suspended in water or buffer have given us results entirely similar to those obtained with *Actinomyces* sp. Namely, lysis of susceptible bacteria in buffer by those organisms has been observed; a lytic protein which dissolves dead bacteria may be obtained from broth-cultures of the proteolytic organisms; and the ether-extract from those same cultures shows a high bactericidal activity in buffer. We have

never been able to obtain the production of new adaptive lysins from mixed cultures in water or water-agar. It then appears to us that some of the relationships which have been described as induced antagonism between proteolytic and peptolytic bacteria should be explained by an entirely different mechanism, identical to the one above suggested for the activity of our *Actinomyces* sp.

CONCLUSIONS

1. Bacteriolysis of living gram-positive bacteria by *Actinomyces* sp. appears to be a complex phenomenon which involves several different factors. First, the susceptible cells must be killed by an ether-soluble substance able to act only when the *Actinomyces* is present or after being artificially extracted. Next, the dead cells are dissolved either by the bacteriolytic enzyme from actinomycetin or by their own autolytic system.

2. A similar explanation may be given for some cases of antagonism exhibited by several proteolytic bacteria. This explanation is entirely different from Schiller's theory of induced antagonism by production of new adaptive lysins.

3. The antagonistic relationships described in this paper must be considered as a weak "natural" antagonism, since the active substances involved are normally found in pure cultures of the antagonists. It takes an intermediate position between strong "natural" antagonism and "direct" antagonism, since the presence of the antagonist is generally needed for its manifestations.

4. The presence in sterile culture-media of bactericidal substances which are precipitated by $(\text{NH}_4)_2\text{SO}_4$ or acidification and may be extracted by organic solvents suggests that at least a part of the toxic agents isolated by various investigators from bacterial cultures in complex media need not be of bacterial origin (Hettche, 1934; Hettche and Weber, 1939; McDonald, 1940; actinomycin B of Waksman and Woodruff, 1940).

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SOME FACTORS WHICH INFLUENCE THE OXIDATION OF SULFHYDRYL GROUPS

By M. L. ANSON

*(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton,
New Jersey)*

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INTRODUCTION

The experiments described in this paper show (1) that the oxidation of SH groups by common oxidizing agents such as ferricyanide and Folin's uric acid reagent is inhibited by cyanide and promoted by copper sulfate, (2) that the SH groups of denatured egg albumin can be oxidized by an equivalent amount of ferricyanide even in the absence of denaturing agents, provided aggregation is avoided, (3) that the SH groups of denatured egg albumin are more easily oxidized by some oxidizing agents in urea or guanidine hydrochloride solution than in a solution of long chain alkyl sulfates¹ or in the absence of denaturing agents, and (4) that the SH groups of egg albumin partially hydrolyzed by pepsin are more easily oxidized than the SH groups of denatured but unhydrolyzed egg albumin. It will be shown in another paper that urea and partial hydrolysis promote the oxidation of protein tyrosine and tryptophane groups as well as protein SH groups and that urea promotes the oxidation even of free tyrosine and tryptophane.

Now that it is clear that the oxidation of SH groups even by common oxidizing agents other than oxygen is dependent on cyanide-sensitive catalysts, the possibility must always be considered that a difference in the ease with which two SH compounds are oxidized may be due, in part at least, to a difference in the catalytic impurities present.

All denaturing procedures bring about the same qualitative changes in a protein's properties. The differences in the ease with which the SH groups of denatured egg albumin are oxidized in the presence of different denaturing agents such as alkyl sulfate, urea, and guanidine hydrochloride, however, show

¹ In a previous note (Anson, 1939a) it was stated that urea and guanidine hydrochloride, like Duponol PC, promote the reaction between ferricyanide and the SH groups of denatured egg albumin but that urea and guanidine hydrochloride are less effective than Duponol PC. By this statement was meant merely that the amount of Duponol PC needed to denature egg albumin and thus to make all the SH groups of denatured egg albumin react with dilute, neutral ferricyanide is much less than the amounts of urea or guanidine hydrochloride needed to achieve the same result.

that the exact reactions of a denatured protein depend on what denaturing agent is present. This dependence has also been shown with the nitroprusside test. The SH groups of denatured egg albumin give a strong pink color with nitroprusside in guanidine hydrochloride solution, a weaker color in urea solution, and a still weaker color in alkyl sulfate solution (Anson, 1941).

It is known from earlier experiments that alkyl sulfate, urea, and guanidine hydrochloride all can denature egg albumin, keep the denatured protein in solution, and make its SH groups accessible to titration. These earlier experiments, however, unlike the present experiments, were carried out in such a way that they did not permit any conclusions about differences in the ease of oxidation of the SH groups of denatured egg albumin in the presence of different denaturing agents. In the earlier experiments the SH groups of the denatured egg albumin were oxidized in neutral solution by relatively strong oxidizing agents such as porphyrindin and ferricyanide. Under such favorable conditions for oxidation the SH groups of denatured egg albumin are oxidized in alkyl sulfate solution as well as in urea solution. They are oxidized, as will be shown, even in the complete absence of substances such as urea and alkyl sulfate provided the denatured protein is prepared with proper precautions. In the present experiments, however, the conditions for the oxidation of the SH groups of denatured egg albumin are made more unfavorable by making the solution more acid or by using a weaker oxidizing agent, the uric acid reagent. Under the new, more unfavorable conditions oxidation of the denatured protein does not take place in the absence of denaturing agents or even in the presence of long chain alkyl sulfates. When urea or guanidine hydrochloride is added or when the protein is digested then oxidation takes place even under the less favorable conditions, and the effects of urea, guanidine hydrochloride, and hydrolysis on denatured egg albumin are thus readily demonstrated. Similarly the effect of added copper sulfate on the oxidation of cysteine by the uric acid reagent is demonstrated by carrying out the oxidation in an acid solution in which no oxidation takes place in the absence of added copper sulfate. In neutral solution, oxidation takes place without added copper sulfate.

The experiments described in this paper have helped to make clearer the factors which influence the oxidation of protein SH groups and they have suggested experiments with other protein groups. By themselves, however, the present experiments are not adequate to decide by what structural mechanisms aggregation, denaturing agents, and hydrolysis influence the SH reactions of egg albumin. Furthermore, although the urea effects which have been observed cannot be imitated by small amounts of copper sulfate, the present experiments do not decide to exactly what extents catalytic impurities contribute to the effects of various reagents on protein SH reactions. Finally, it is not known to what extent the uric acid reagent influences the surface structure and aggregation of denatured egg albumin.

EXPERIMENTAL RESULTS

Cyanide.—The oxidation of the SH groups of cysteine (Mathews and Walker, 1909) and denatured egg albumin (Rosenthal and Voegtlin, 1933) by oxygen is promoted by heavy metal compounds, especially copper salts, and indeed does not take place at all in the absence of heavy metal catalysts (Warburg and Sakuma, 1923). The work on the heavy metal catalysis of oxidation of SH by oxygen has recently been reviewed by Bernheim and Bernheim (1939).

Most commercial samples of guanidine hydrochloride contain impurities which catalyze the oxidation of the SH groups of denatured egg albumin by oxygen. This oxidation can be inhibited by cyanide (Anson, 1941). When the attempt was made to titrate the SH groups of denatured egg albumin in guanidine hydrochloride solution with ferricyanide in the presence of cyanide, added to prevent oxidation by oxygen, it was found that the added cyanide inhibited not only the oxidation of SH by oxygen but also stopped much of the oxidation of SH by ferricyanide. Cyanide also slowed up but did not prevent the oxidation of cysteine by ferricyanide (Anson, 1941). This inhibition of ferricyanide oxidation by cyanide indicated that even the ferricyanide oxidation requires heavy metal catalysts and led to the present experiments with cyanide and copper sulfate.

The oxidations of SH by the uric acid reagent described in this paper are more completely inhibited by cyanide than the ferricyanide oxidations previously studied. 1 drop of 0.1 N K CN in 10 cc. solution inhibits completely the oxidation of cysteine by neutral uric acid reagent. Somewhat more cyanide is needed to inhibit the oxidation of the SH groups of denatured egg albumin in neutral urea solution (Table I).

Heavy Metals.—Copper sulfate promotes the oxidation of cysteine by the uric acid reagent and ferricyanide.

The oxidation of cysteine by the uric acid reagent which takes place in alkaline solution (Folin and Looney, 1922) and in neutral solution (Lugg, 1932; Mirsky and Anson, 1935) does not take place at pH 4.8. If copper sulfate is added, however, the oxidation takes place even at pH 4.8 (Table I).

Similarly, in neutral solution 1 cc. of 0.001 M ferricyanide is reduced by 1 cc. of 0.001 M cysteine with disappearance of the brown color of ferricyanide. At pH 4.8 the brown color does not disappear. If copper sulfate is added at pH 4.8, however, the brown color of ferricyanide is replaced by the weak red color of copper ferrocyanide.

How acid the solution of cysteine has to be made to prevent oxidation by the uric acid reagent or ferricyanide varies with different samples of cysteine. Presumably different samples of cysteine contain different amounts of catalytic impurities. The sample of cysteine used for the experiments in Table I was not the most easily oxidized or the least easily oxidized of the samples of cysteine I have encountered. If the reagents were completely free of heavy metal impurities, oxidation would probably not take place even in neutral solution and even a trace of added copper sulfate would promote oxidation.

In comparing the oxidation of cysteine under different conditions it is important to keep the volume of solution in which the reaction takes place constant. At pH 5.2 cysteine is oxidized by the uric acid reagent 15 per cent if the reaction is carried out for 10 minutes in 10 cc. of solution. If, however, the reaction is carried out in 3 cc. with the same absolute amounts of reactants and the solution is diluted to 10 cc. at the end of 10 minutes, then 71 per cent of the cysteine is oxidized. In 6 cc. of reaction solution, the oxidation is 30 per cent complete.

TABLE I
Effect of CN and CuSO₄ on Oxidation of SH Groups

10⁻³ mM of cysteine or 10 mg. egg albumin plus 5.9 gm. urea in 10 cc. of 0.1 M phosphate or acetate solution. Oxidation for 10 minutes at 25°C. by 0.5 cc. of uric acid reagent

SH compound	pH	CuSO ₄	KCN	Oxidation
		mM × 10 ³	mM × 10 ³	per cent
Cysteine.....	6.6	0	0	100
Cysteine.....	6.6	0	5	0
Albumin.....	6.9	0	0	100
Albumin.....	6.9	0	10	39
Albumin.....	6.9	0	30	10
Cysteine.....	4.8	0	0	0
Cysteine.....	4.8	4	0	88
Cysteine.....	4.8	1	0	46

Denaturing Agents.—The SH groups of denatured egg albumin have been estimated in a variety of ways in solutions of alkyl sulfate, urea, and guanidine hydrochloride which serve to denature the protein and to keep the denatured protein in solution. In a neutral solution of guanidine hydrochloride it requires 1 cc. of 0.001 M porphyrindin to abolish the SH groups of 10 mg. of coagulated egg albumin as shown by the abolition of the nitroprusside test (Greenstein, 1938). In a neutral solution of Duponol PC, a mixture of long chain alkyl sulfates, 1 cc. of 0.001 M ferrocyanide is formed when 1 cc. of 0.001 M ferricyanide is added to 10 mg. of denatured egg albumin (Anson, 1939*a*, *b*). The evidence that ferricyanide is oxidizing only SH groups and all the SH groups is that cysteine is the only amino acid known to reduce ferricyanide under the conditions used, that heat denatured egg albumin treated with iodoacetamide in the absence of denaturing agents no longer reduces ferricyanide in Duponol PC solution, that the amount of ferricyanide reduced by albumin denatured by Duponol PC is within wide limits independent of the concentration of ferricyanide, and the pH, time, and temperature of the reaction (Anson, 1939*a*, *b*), that the amount of ferricyanide reduced is the same whether the reaction is carried out in solution of Duponol PC, urea, or guanidine hydrochloride (Anson, 1940, 1941), and that the number of SH groups detected is the same whether one measures the amount of ferricyanide reduced or the amount of ferricyanide, tetrathionate, or *p*-chloromercuribenzoate needed to abolish the SH groups, as shown by the abolition of the nitroprus-

side test (Anson, 1940, 1941) or the number of SH groups of neutral native egg albumin titrated by iodine in 1 M KI at 0°C. (Anson, unpublished experiments).²

Before reagents such as guanidine hydrochloride and alkyl sulfate were introduced, attempts were made to estimate the SH groups of heat coagulated egg albumin which led to SH values that are now known to be about half the correct value. Hopkins (1925) first showed that oxidized glutathione can oxidize some, at least, of the SH groups of denatured egg albumin. When cystine (Mirsky and Anson, 1935) and porphyrindin (Kuhn and Desnuelle, 1938) were added to 10 mg. of coagulated egg albumin, the amount of oxidizing agent reduced was equivalent to only 1 cc. of 0.0005 M cysteine.

The first studies of the reactions between ferricyanide and coagulated SH proteins showed that ferricyanide can oxidize tyrosine and tryptophane groups as well as SH groups and that the amount of ferricyanide reduced is greater the higher the concentration of ferricyanide (Mirsky and Anson, 1936). Later experiments showed that if the concentration of ferricyanide is not too high, ferricyanide reacts only with the SH groups of denatured egg albumin and not with tyrosine, tryptophane, or disulfide groups (Anson, 1939*b*), and that the amount of ferricyanide reduced by SH groups alone depends on the concentration of ferricyanide and on the physical state of the protein, the more the protein is aggregated, the less ferricyanide being reduced (Anson, 1939*a*).³ Reference was made to earlier viscosity measurements which showed

² There is no significant change in the sedimentation rate of the albumin as a result of the iodine reaction. Thus the oxidation of the SH groups of native egg albumin by an equivalent amount of iodine does not involve polymerization of the protein. The SH groups of egg albumin can be abolished by reaction of the neutral native protein not only with iodine but also with dilute permanganate or with hydrogen peroxide in thousands of times greater concentration than is needed for the oxidation of the SH groups of denatured egg albumin.

³ When a substance reacts with a protein which is precipitated instead of in solution, the substance has farther to diffuse before it meets a protein particle, it has to penetrate the precipitate, and it reacts with groups whose properties must be changed to some extent by the bonding between molecules in the precipitate. Since a significant fraction of all cellular proteins is not in solution, it would be desirable that the reactions of proteins in the solid state be systematically studied. Some years ago, as a result of some failures in trying to dry crystalline carboxypeptidase without inactivation, I began a study of the denaturation of protein in the solid state. When protein crystals were made insoluble by denaturation procedures the superficial crystalline form was retained (Anson, 1938). The temperature coefficient of denaturation of protein in the solid state was roughly the same as that of denaturation in solution (unpublished experiments). If a solid particle had denatured as a whole, the way a single protein molecule in solution denatures as a whole, the temperature of denaturation would have been almost as sharp as a melting point. When the SH groups of proteins denatured in the solid state were studied by the techniques then available, difficulties were encountered which led to a further study of SH groups. The subject of the SH groups of protein denatured in the solid state has not since been reinvestigated.

that denatured egg albumin can be aggregated even in apparently clear solution and that this aggregation is sensitive to salt (Anson and Mirsky, 1932). Because of the aggregation effect the practical estimation of the SH groups was carried out in the presence of Duponol PC, which acts as a solvent as well as a denaturant. Furthermore, the experiments on the reaction of ferricyanide with the SH groups of denatured egg albumin in the absence of denaturing agents were carried out not with a precipitate but with a solution of denatured egg albumin which reacted much more readily with ferricyanide than the suspensions of coagulated egg albumin previously used. Denaturation was brought about in acid solution and then the denatured protein was cooled and brought into neutral solution. The SH groups of 10 mg. of such a preparation of denatured egg albumin in solution were oxidized 64 per cent by 1 cc. of 0.001 *M* ferricyanide and oxidized 94 per cent if the ferricyanide concentration was increased 25 times (Anson, 1939*b*). For the present experiments on heat denatured egg albumin in the absence of denaturing agents, denatured egg albumin was prepared in essentially the same way as previously but even more care was taken to avoid precipitation.

The effect of urea on the SH groups of denatured egg albumin can be shown with the uric acid reagent in neutral solution or with ferricyanide in acid solution. The uric acid reagent oxidizes the SH groups of denatured egg albumin in neutral urea solution (Table II, Experiment 1) but not in alkyl sulfate solution (Experiment 2) or in the absence of denaturing agents (Experiment 3). Guanidine hydrochloride gives a precipitate with the uric acid reagent.

Ferricyanide, a stronger oxidizing agent than the uric acid reagent, oxidizes the SH groups of neutral denatured egg albumin even in alkyl sulfate solution⁴ (Table II, Experiment 4) or in the absence of denaturing agents (Experiments 5 and 6). In alkyl sulfate solution at pH 4.9, however, ferricyanide oxidizes the SH groups of denatured egg albumin only 21 per cent (Experiment 7). The per cent oxidation is increased from 21 to 86 if urea is present instead of alkyl sulfate at pH 4.9 (Experiment 8). In the absence of denaturing agents denatured egg albumin is insoluble at pH 4.9. At pH 3.9 oxidation by ferricyanide is more complete in guanidine hydrochloride than in urea solution (Experiments 9 and 10).

The blue color formed when the SH groups of a denatured protein are oxidized by the uric acid reagent in neutral urea solution is a convenient measure of the SH content of the protein. The number of SH groups of completely denatured egg albumin, edestin, and tobacco mosaic virus oxidized

⁴ The SH groups of denatured tobacco mosaic virus in alkyl sulfate solution are not oxidized even by ferricyanide. Recently Dr. Seymour Cohen has found by measurements of sedimentation rates that tobacco mosaic virus in sodium dodecyl sulfate solution is dissociated into molecules about the size of egg albumin. The failure of ferricyanide to oxidize the virus SH groups in sodium dodecyl solution is therefore not due to aggregation.

in neutral urea solution by the uric acid reagent is the same as the number of SH groups detected by ferricyanide titration in guanidine hydrochloride solution by the procedure described elsewhere (Anson, 1941; Anson and Stanley, 1941). Egg albumin and edestin are rapidly denatured in neutral urea

TABLE II

Effect of Denaturing Agents on the Oxidation of the SH Groups of 10 Mg. of Denatured Egg Albumin

Experiment	Denaturant	pH	Oxidant	Oxidation in 10 minutes	Composition of solution				
					Amount of denaturant	Buffer mixture	Amount of buffer	Final volume	Amount of oxidant
				per cent		ratio of 1 M salts	cc.	cc.	
1	Urea	6.6	Uric acid reagent	100	5.9 gm.	$\frac{1 \text{ Na}_2\text{HPO}_4}{3 \text{ NaH}_2\text{PO}_4}$	1	10	0.5 cc.
2	Sodium dodecyl sulfate	6.7	" "	0	10 mg.	$\frac{1 \text{ Na}_2\text{HPO}_4}{1 \text{ NaH}_2\text{PO}_4}$	1	6	0.5 cc.
3	Heat	6.7	" "	Trace	0	$\frac{1 \text{ Na}_2\text{HPO}_4}{1 \text{ NaH}_2\text{PO}_4}$	0.1	6	0.5 cc.
4	NaDS	6.7	Ferricyanide	100	10 mg.	$\frac{1 \text{ Na}_2\text{HPO}_4}{1 \text{ NaH}_2\text{PO}_4}$	1	10	$50 \times 10^{-3} \text{ mM}$
5	Heat	6.7	" "	94	0	$\frac{1 \text{ Na}_2\text{HPO}_4}{1 \text{ NaH}_2\text{PO}_4}$	0.1	10	10^{-3} mM
6	Heat	6.7	" "	100	0	$\frac{1 \text{ Na}_2\text{HPO}_4}{1 \text{ NaH}_2\text{PO}_4}$	0.1	10	$50 \times 10^{-3} \text{ mM}$
7	NaDS	4.9	" "	21	10 mg.	$\frac{1 \text{ HAc}}{2.5 \text{ NaAc}}$	0.5	1.6	$50 \times 10^{-3} \text{ mM}$
8	Urea	4.9	" "	86	1.5 gm.	$\frac{4 \text{ HAc}}{1 \text{ NaAc}}$	0.5	2.5	$50 \times 10^{-3} \text{ mM}$
9	Urea	3.9	" "	52	1.5 gm.	(2 N HAc)	0.5	2.5	$50 \times 10^{-3} \text{ mM}$
10	Guanidine HCl	3.9	" "	82	1.5 gm.	$\frac{4 \text{ HAc}}{1 \text{ NaAc}}$	0.5	2.5	$50 \times 10^{-3} \text{ mM}$

solution in the presence of the uric acid reagent. Tobacco mosaic virus, however, is not rapidly denatured by neutral urea and the native virus does not reduce the uric acid reagent in urea solution. The virus, therefore, is denatured in acid urea solution before the estimation of the SH by the uric acid reagent in neutral solution.

Greenstein (1938, 1939) titrating with porphyrindin, a much stronger oxidizing agent than the uric acid reagent, found that fewer SH groups of egg albumin, edestin,

and globin were made titratable by urea than by guanidine hydrochloride. In the light of the present experiments it is unlikely that, as Greenstein supposed, urea failed to "liberate" all the SH groups of his proteins. Denatured SH proteins give a much weaker pink color with nitroprusside in urea than in guanidine hydrochloride solution (Anson, 1941). It is therefore possible that a negative nitroprusside test is not a suitable end-point for the porphyrindin titration in urea solution.

Urea makes the pH of buffers more alkaline. This effect is especially marked with acetate buffers. When a comparison is made between an oxidation in the presence and absence of urea, therefore, a more acid buffer is used in the presence than in the absence of urea so that the pH, as measured by the glass electrode, is the same in both cases (Table II, Experiments 7 and 8).

When urea or guanidine hydrochloride is used a considerable part of the total volume of solution is occupied by the denaturing agent. Thus for a given total volume of solution the concentration of reactants per gram of water is greater in the presence than in the absence of urea and guanidine hydrochloride. An increase in concentration of reactants per gram of water, however, favors oxidation, as has been pointed out. Thus when a comparison is made of a particular oxidation in the presence and absence of urea and guanidine hydrochloride, the reaction is carried out at constant concentrations of reactants per gram of water not per cubic centimeter of solution. The exact amount of water in a saturated urea solution which is available for a protein reaction is not known.

The uric acid reagent not only does not oxidize the SH of denatured egg albumin in neutral alkyl sulfate solution itself but interferes with the oxidation by ferricyanide in alkyl sulfate solution. Tungstate does not interfere with the ferricyanide oxidation. Both the uric acid reagent and tungstic acid, however, slow up the reaction between ferrocyanide and ferric sulfate to form Prussian blue. No study was made of the mechanisms of these inhibitions.

Alkyl sulfates inhibit the oxidation of the SH groups of denatured egg albumin in urea solution by the uric acid reagent. This inhibition is greater the higher the concentration of alkyl sulfate, and it is greater with Duponol PC, a mixture of long chain alkyl sulfates, than with pure sodium dodecyl sulfate. Thus detergents may act as inhibitors as well as solvents.

Hydrolysis.—The effect of hydrolysis in activating the SH groups of denatured egg albumin can be demonstrated with either the uric acid reagent or with ferricyanide. Denatured egg albumin is not oxidized by the uric acid reagent in neutral solution in the presence or in the absence of alkyl sulfate (Table II, Experiments 2 and 3). In contrast, all the SH groups of a peptic hydrolysate of egg albumin are oxidized by the uric acid reagent in neutral solution either in the presence or in the absence of alkyl sulfate (Table III).

At pH 4.8 in a solution of sodium dodecyl sulfate, 1 cc. even of 0.05 M ferricyanide oxidizes 10 mg. of egg albumin only 15 per cent. Under the same conditions the hydrolysate is oxidized 83 per cent (Table III).

78 per cent of the SH in a peptic digest of egg albumin is precipitated by the uric acid reagent, which is a phospho-18-tungstic acid (Wu, 1920), or by saturated ammonium sulfate. The SH which is precipitated is either a part of peptides precipitable by phospho-18-tungstic acid (a more satisfactory precipitant for peptides than tungstic acid) or it is adsorbed to the precipitate which is formed. Free cysteine and cysteine added to a peptic digest of egg albumin are not precipitated by phospho-18-tungstic acid. The 22 per cent of the SH of a peptic digest of egg albumin which is not precipitated by phos-

TABLE III
Oxidation of SH Groups of 10 Mg. Egg Albumin Digested by Pepsin
10 minutes oxidation in 10 cc. of 0.1 M phosphate or acetate solution

Oxidant	Amount of oxidant	pH	Sodium dodecyl sulfate	Oxidation
			mg.	per cent
Uric acid reagent	0.5 cc.	6.8	0	100
Uric acid reagent	0.5 cc.	6.8	50	99
Ferricyanide	10^{-3} mM	6.8	0	99
Ferricyanide	10^{-3} mM	6.8	50	100
Ferricyanide	50×10^{-3} mM	4.8	10	83

pho-18-tungstic acid is either free cysteine or SH peptides which are not precipitated by phospho-18-tungstic acid.

Pure SH peptides such as would be desirable for studies of the relation of peptide structure to the properties of the peptides' SH groups have as yet not been isolated from enzymatic digests of proteins. The ease with which SH or its oxidation product, S—S, is estimated should facilitate the isolation of such peptides.

If hydrolyzed egg albumin is allowed to stand in neutral solution, the SH groups are oxidized to S—S by the oxygen of the air. As will be described in a later paper, the total S—S present can then be readily estimated by sulfite plus the uric acid reagent.

EXPERIMENTAL PROCEDURES

Reagents.—Cysteine hydrochloride—Hoffmann-La Roche. Dissolved in 0.1 N HCl, stored at 0°C., and used promptly.

Urea.—Merck's reagent. The SH groups of denatured egg albumin were stable for one-half hour in the presence of the particular sample of urea used. Other samples contained more catalytic impurities.

Guanidine hydrochloride—prepared from purified guanidine carbonate according to Anson (1941).

Duponol PC—du Pont. Stored as a 10 per cent solution.

Sodium dodecyl sulfate—prepared especially by du Pont through the kind offices of Dr. Samuel Lenher. Not available commercially.

Ferricyanide—purified according to Anson (1941).

Uric acid reagent—prepared according to Folin (1934).

Ferric sulfate with gum ghatti—prepared according to Folin and Malmros (1929).

Egg Albumin.—Recrystallized by ammonium sulfate or sodium sulfate to constant SH content, dialyzed, and stored frozen. Once crystallized egg albumin always has a low SH content and sometimes the SH content is not raised to the usual value of 1.2 per cent cysteine even by repeated recrystallizations. When two procedures for the estimation of SH are being compared, the same sample of egg albumin should be used for both procedures.

Egg albumin denatured by heating in acid is prepared as follows. To 6 cc. of 2 per cent egg albumin add 1 cc. of 1 *N* HCl, place in 50°C. bath for 5 minutes, cool in ice water, add slight excess of 1 *N* NaOH so that the solution is blue to thymophthalein (about 0.1 cc. 0.1 *N* NaOH excess per 10 mg. albumin), add water to 12 cc., cool and store in ice water, and use promptly. Egg albumin denatured by trichloroacetic acid and washed by decantation before solution with NaOH gives the same results as egg albumin denatured at 50°C.

To prepare digested egg albumin a pH 2.0 solution containing 10 mg. of egg albumin and 1 mg. of crystalline pepsin per cc. of 0.045 *N* HCl is kept at 65°C. for 1 hour. The vessel is evacuated and filled with 99.8 per cent nitrogen several times before digestion is begun. If digestion is carried out in air, there is a small loss in SH.⁵ The hydrolysate gives no precipitate with hot 0.2 *N* trichloroacetic acid. Pepsin itself self-digested at pH 2.0 and 65°C. does not give a nitroprusside test in guanidine hydrochloride solution.

The quantities of reagents used, the volume of solution in which the reaction is carried out, the time of reaction, and the percentage oxidation are given in the tables. The pH was measured by the glass electrode.

The reactions with the uric acid reagent are all carried out at 25°C. After the designated time of reaction the solution is diluted to 10 cc., if it already is not 10 cc., and the blue color formed by the reduction of the uric acid reagent is read either against a standard blue solution formed by the oxidation of 1 cc. of 0.001 *M* cysteine or against a blue glass calibrated with a standard blue solution. When the blue solution contains urea it is necessary in order to have an optically homogeneous solution either to use a dry colorimeter cup or, if the cup contains residual water, to rinse the cup with a urea solution containing 1 gm. of urea per cc. water.

⁵ When egg albumin was hydrolyzed completely by hot, concentrated sulfuric acid in air, only 1 cc. of 0.005 *M* cysteine was found in the hydrolysate of 10 mg. of egg albumin (Mirsky and Anson, 1935). Half the SH was presumably lost by oxidation and by adsorption to humin. During the partial hydrolysis by pepsin under the conditions described there is no oxidation of SH or formation of humin.

When SH is oxidized by the uric acid reagent, the solutions are prepared and mixed as follows. Into one test tube there are pipetted the uric acid reagent, the 1 cc. of 1 M buffer (except with heat denatured albumin, when 1 cc. of 0.1 M buffer is used), a volume of 0.5 N NaOH equal to the volume of uric acid reagent (this NaOH being added to neutralize the acid reagent), copper sulfate or alkyl sulfate, when they are used, and water to make up a volume 1 cc. less than the final volume desired. 1 cc. of SH solution is pipetted into another test tube. The uric acid reagent solution is poured into the SH solution and the mixed solution poured back and forth. This manner of mixing minimizes formation of turbid solutions and the introduction of catalytic impurities.

When urea is used, it is first placed into a dry test tube to which the uric acid reagent, etc., are then added. The test tube is placed in a 37°C. bath to dissolve the urea and then the solution is brought to 25°C. before being mixed with the SH solution. After the mixing, the mixed solution is centrifuged to hasten the removal of air bubbles.

When both Duponol PC and 5.9 gm. urea are present in 10 cc. solution, some of the Duponol PC comes out of solution in time. Before the colorimetric reading is made, therefore, the solution is warmed to dissolve the Duponol.

When cyanide is added to inhibit oxidation by the uric acid reagent, it is added to the SH solution just before the mixing with the uric acid reagent.

The ferricyanide reactions are all carried out at 37°C. and, except Experiments 7-10 of Table II, are carried out as follows. The reagents and water are mixed in a test tube, the SH solution being added next to last, and the ferricyanide last. 1 cc. of 1 M buffer is used, except with heat denatured egg albumin when 1 cc. of 0.1 M buffer is used. In the experiments with heat denatured egg albumin, furthermore, the ferricyanide is added promptly after the protein. If the heat denatured protein is allowed to stand in the neutral buffer solution, its SH groups become less completely oxidized by dilute ferricyanide. Ferricyanide is added last so that the alkaline heat denatured albumin does not come in contact with the ferricyanide before the protein is neutralized. After the ferricyanide reaction has taken place in 10 cc. for the designated time at 37°C., there are added 0.5 cc. of 2 N H₂SO₄, 0.5 cc. of 10 per cent Duponol PC, 0.5 cc. of 0.1 M ferricyanide or 0.5 cc. of water if the solution already contains concentrated ferricyanide, and 0.5 cc. of ferric sulfate. As previously pointed out (Anson, 1939*b*), the Duponol is added to prevent turbidity and the extra ferricyanide to promote the formation of Prussian blue. After 20 minutes the Prussian blue formed is read in red light against either a Prussian blue solution developed from 1 cc. of 0.001 M ferrocyanide or against a blue glass calibrated with a Prussian blue standard.

Experiments 7-10 of Table II are carried out as follows. To a mixture of 0.5 cc. SH solution, 0.5 cc. 1 M buffer, and 0.5 cc. ferricyanide there is added either 0.1 cc. of 10 per cent sodium dodecyl sulfate, 1.5 gm. of urea, or 1.5 gm. of guanidine hydrochloride. The ferrocyanide formed in the alkyl sulfate and urea solutions is estimated as Prussian blue as already described, the solution being diluted to 10 cc. before the addition of ferric sulfate, etc. Guanidine hydrochloride, however, interferes with the complete conversion of ferrocyanide into Prussian blue. So in the experiment with guanidine hydrochloride one measures not the ferrocyanide formed but

the number of SH groups surviving. The protein is precipitated and washed with 0.2 M trichloroacetic acid and the protein's SH groups estimated with ferricyanide in neutral Duponol PC solution.

The experiment on the effect of copper sulfate on the oxidation of cysteine by ferricyanide is carried out as follows. To 1 cc. of 0.001 M cysteine there are added 0.3 cc. of a 1 M buffer containing equal parts acetic acid and sodium acetate, and 0.5 cc. of 0.002 M ferricyanide. The brown color of ferricyanide does not disappear. 5 drops of 0.002 M copper sulfate are added. The brown color disappears immediately and is replaced by the weak red of copper ferrocyanide.

The experiment which shows that 78 per cent of the SH of a peptic digest of egg albumin is precipitated by 0.05 M phospho-18-tungstic acid in 1 N H₂SO₄ is carried out as follows. To 2 cc. digest there are added 0.8 cc. uric acid reagent, 5 cc. 2 N H₂SO₄, and water to 10 cc. The precipitate is centrifuged off and its SH content estimated with the uric acid reagent in neutral urea solution. The SH content of the precipitate is the same whether 1 cc. of 0.001 M cysteine is added to the digest or not.

SUMMARY

1. Cyanide inhibits the oxidation of the SH groups of cysteine and denatured egg albumin by the uric acid reagent.
2. At pH 4.8 cysteine is oxidized by the uric acid reagent and by ferricyanide in the presence but not in the absence of added copper sulfate.
3. In neutral solution, the uric acid reagent oxidizes the SH groups of denatured egg albumin in the presence of urea but not in the presence of alkyl sulfate or in the absence of denaturing agents.
4. Ferricyanide oxidizes the SH groups of neutral denatured egg albumin even in the presence of alkyl sulfate or, if precautions are taken to avoid aggregation, in the absence of denaturing agents.
5. In acid solution, ferricyanide does not oxidize the SH groups of denatured egg albumin completely. The oxidation is more complete, however, in the presence of urea than in the presence of alkyl sulfate, and more complete in the presence of guanidine hydrochloride than in the presence of urea.
6. The uric acid reagent which does not oxidize the SH groups of neutral denatured but unhydrolyzed egg albumin in the absence of denaturing agents does, under the same conditions, oxidize the SH groups of egg albumin partially hydrolyzed by pepsin.
7. At pH 4.8 in alkyl sulfate solution ferricyanide oxidizes the SH groups of digested egg albumin more completely than the SH groups of denatured but undigested egg albumin.

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PURIFICATION AND CRYSTALLIZATION OF DIPHTHERIA ANTITOXIN

By JOHN H. NORTHROP

*(From the Laboratories of The Rockefeller Institute for Medical
Research, Princeton, New Jersey)*

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The results of a series of investigations of the chemical nature of antibodies and antitoxins (Avery, 1915), (Gay and Chickering, 1915), (Felton, 1932), (Chow and Goebel, 1935), (Chow and Wu, 1937), (Kirk and Sumner, 1931, 1934), (Heidelberger and Kendall, 1936), (Pope, 1938), (Pope and Healey, 1939), (Petermann and Pappenheimer, 1941) have shown that these substances are proteins closely related to the normal serum proteins. Since they possess special properties not exhibited by the normal serum proteins they must have some special chemical structure but the nature of this structure still remains entirely unknown. The first step in an attempt to determine the structure is the isolation in pure form of the antitoxin. Chow, Goebel, Heidelberger, and their coworkers have obtained pneumococcus antibodies which were pure in the sense that they were completely precipitated by the specific carbohydrate. Petermann and Pappenheimer (1941) have isolated diphtheria antitoxin which was homogeneous by electrophoresis and ultracentrifuge but was not completely precipitated by diphtheria toxin.

Many antibodies form precipitates with their antigens and dissociation of this precipitate has frequently been used as a method of purification. Ramon (1922) found that diphtheria toxin precipitates when mixed in certain proportions with diphtheria antitoxin and was able (1923) to obtain some antitoxin by dissociating the complex in slightly acid solution. Pope and Healey (1939) and Petermann and Pappenheimer (1941) dissociated the complex by digestion with pepsin¹ at pH 3.0.

Pepsin attacks the antitoxin (Parfentjev, 1937) as well as the toxin and it seemed possible that a more homogeneous preparation might be obtained by using trypsin which, so far as is known, attacks only the toxin. If trypsin is added to a solution of the toxin-antitoxin complex in dilute acid and the solution then neutralized the toxin is destroyed and 30-60 per cent of the original antibody may be recovered. The antibody recovered in this way is 90 per cent or more precipitated by toxin; it is homogeneous in the ultracentrifuge or

¹ Preliminary experiments indicate that tetanus antitoxin may be purified in this way.

in the electrophoresis cell. The molecular weight of this antibody is 90,500 (Rothen, 1941) while antibody which has not been treated with trypsin has a higher molecular weight. Trypsin, therefore, digests part of the antitoxin as well as the toxin just as does pepsin (Petermann and Pappenheimer, 1941). This preparation corresponds closely to that obtained by Petermann and Pappenheimer. The solubility of this preparation is not constant, however, but varies with the amount of solid showing that at least two proteins are present, both of which react with toxin. The preparation may be further purified by fractional precipitation with ammonium sulfate. The most soluble fraction has constant solubility and appears to be a pure protein. It crystallizes readily in poorly formed thin plates. This protein is 90 per cent or more precipitated by diphtheria antitoxin and has about 700-900 antitoxin units per milligram protein nitrogen by the flocculation test and about 700 units per milligram by the animal protection test.

The preparation is homogeneous by electrophoresis or ultracentrifuge (Rothen, 1941). The protein is quite unstable and changes rapidly into a slightly less soluble form which does not crystallize and has a longer flocculation time but which has about the same antitoxic titer. This form is stable between pH 3.0 and 9.0. The carbohydrate content, calculated as glucose, is about 3 per cent.

Precipitation of purified antitoxin and crude toxin takes place over a wider range of concentrations than does the precipitation of crude antitoxin. Precipitation of pure antitoxin with purified toxin takes place over a very wide range and the soluble zone in the region of toxin excess disappears.

The toxin-antitoxin complex is soluble in the absence of salt and in solutions more alkaline than 7.0. This solution is precipitated by positive ions. The concentration of ions required to precipitate decreases as the valence increases. The reaction of toxin plus antitoxin, therefore, consists of two steps, as does the agglutination of bacteria. The first step is the formation of a compound. This is the specific part of the reaction. The precipitation of this compound then depends upon the salt concentration and hydrogen ion in the same way as does the precipitation of other colloidal suspensions.

The purified antitoxin does not precipitate with serum of rabbits immunized against normal horse serum. Guinea pigs sensitized with purified antibody react when injected with very small amounts of antibody but require very large amounts of normal serum to cause shock.

Experimental Results

1. *Action of Trypsin on Toxin and Antitoxin.*—Diphtheria toxin is rapidly digested by trypsin whereas antitoxin is not. If trypsin were added to a solution of toxin-antitoxin, therefore, it would be expected that the toxin would be digested and that the complex would then dissociate until all the toxin was destroyed. This does not occur but instead there is a slow formation of non-protein nitrogen with no

TABLE I

Digestion of Toxin-Antitoxin with Trypsin at pH 8.0

100 ml. antidiphtheria plasma (500 L_f /ml.) mixed with 1 liter diphtheria toxin (50 L_f /ml.). Stood 25°C. 24 hrs., decanted. Precipitate suspension centrifuged and precipitate washed three times with $m/20$ pH 7.4 phosphate buffer. Washed twice with water. Precipitate stirred with 100 ml. water. Gelatinous mass dissolved slowly to slightly opalescent solution. Titrated to pH 8.0 with NH_4OH . 0.05 mg. trypsin $N/ml.$ added. Stood at 25°C. Analyzed for non-protein nitrogen. 5 ml. + 0.5 ml. $m/2$ pH 7.4 phosphate. Stood 25°C. for $\frac{1}{2}$ hour and filtered. Filtrate analyzed for antitoxin.

Time at 25°C.	Protein nitrogen/ml.	Non-protein nitrogen/ml.	L_f /ml. filtrate after precipitation at pH 7.4
	mg.	mg.	
0	0.96	0.06	0
18 hrs.	0.70	0.30	<40
Same solution, boiled before addition of trypsin			
0	0.90		
10 min.	0.40		
1 hr.	0.30		
24 hrs.	0.20		

TABLE II

Toxin-Antitoxin + Acetic Acid pH 4.0 (No Trypsin)

	No.	Vol.	PN/ml.	L_f /ml.	Total	L_f/PN
			mg.			
200 ml. antidiphtheria plasms (500 L_f /ml.) + 2000 ml. toxin (45 L_f /ml.) at 25°C. for 24 hrs. Decanted. Precipitate suspension + 10 gm. Hyflo. Filtered and washed two times with $m/10$ pH 7.4 phosphate, washed two times with water. Precipitate suspended in 300 ml. water + 13 ml. 2 N acetic acid and filtered. Filtrate clear, pH 4.0-4.1 (brom cresol green) 24 hrs., 5°C.....	1	200	1.0	(450)	(90,000)	
50°C. 20 hrs. + 200 ml. water + 6 ml. 5 N NH_3 (pH 7.2), precipitate + Hyflo. Filtered. Filtrate.....	2	400	0.04	30	12,000	700
400 ml. No. 2 + 150 ml. saturated ammonium sulfate. Precipitate. Stood 25°C. + 3 gm. Hyflo. Filtered. Precipitate + $n/10$ pH 7.4 phosphate.....	3	50		200	10,000	
50 ml. No. 3 + 20 ml. saturated ammonium sulfate (0.35 saturated) slight precipitate + Hyflo. Filtered. Precipitate + phosphate.....	4	10	$\begin{cases} 0.26 \\ 0.32 \end{cases}$	$\begin{cases} 200 \\ 150 \end{cases}$	2,000	500
Filtrate.....	5	70	0.16	150	10,000	900

measurable liberation of antitoxin (Table I). If the toxin-antitoxin solution is boiled before the addition of trypsin it is rapidly digested. These results show that the toxin-antitoxin is not a denatured protein and suggest that the complex does not dissociate at pH 7.0-8.0.

Pappenheimer has shown that diphtheria toxin is unstable in acid and is rapidly denatured on the acid side of pH 6.0 and this observation has been confirmed in the course of this work. The toxin-antitoxin complex dissolves on the acid side

TABLE III

Digestion of Toxin-Antitoxin from Crude or Purified Antitoxin at pH 3.5 and 7.4, 25°C.

Toxin-antitoxin from plasma. Prepared same as in Table II except not washed with distilled water. Suspended in N/10 pH 7.4 phosphate. 0.30 mg. P.N./ml.

Toxin-antitoxin from purified antibody. 5000 L_f units toxin mixed with 5000 units purified antitoxin. Precipitate decanted and washed with N/10 pH 7.4 phosphate. Suspended in N/20 pH 7.4 phosphate. Total N/ml. 0.16 mg.

20 ml. of suspension in N/10 pH 7.4 phosphate buffer titrated to pH 3.7 + N/10 hydrochloric acid. 0.05 mg. trypsin N/ml. added. Analyzed for non-protein nitrogen. Titrated to pH 7.2, stood 2-7 hrs. Filtered. Filtrate analyzed for non-protein nitrogen and antitoxin.

Time	pH	Toxin-antitoxin from plasma and toxin				Toxin-antitoxin from purified antibody			
		Total N/ml.	Non-protein N/ml.	P.N./ml.	L_f /ml.	Total N/ml.	Non-protein N/ml.	P.N./ml.	L_f /ml.
hrs.		mg.	mg.	mg.		mg.	mg.	mg.	
0	3.7	0.38	0.05	0.33	(200 equivalent)	0.16	0.06	0.10	(200 equivalent)
2.5	3.7		0.08	0.30			0.06	0.10	
			Titrated to pH 7.2						
0	7.2		0.08	0.30			0.06	0.10	
1	7.2		0.20	0.18			0.065	0.095	
3	7.2		0.19	0.19			0.075	0.085	
		Heavy precipitate.		Filtered.	Filtrate	Very slight precipitate.		Filtered.	Filtrate
	7.2	0.26	0.19	0.07	80	0.16	0.07	0.09	80
		Filtrate from control solution, no trypsin				Control solution, no trypsin			
			0.05	0	0			0.04	40

of about pH 4.7 and if it were dissociated it would be expected that the toxin would be denatured and the antitoxin liberated. When crude toxin is precipitated with antitoxin and the entire suspension made acid and then alkaline, no precipitate appears, indicating that the toxin may have been destroyed. If the experiment is repeated with washed toxin-antitoxin in buffer, however, the precipitate reappears on neutralization. It is possible to recover some toxin by heating such an acid solution of toxin-antitoxin, as Ramon stated (Table II) but the yield is quite low. However, if trypsin is added to such an acid solution of toxin-antitoxin formed from either pure or crude antitoxin and the solution then neutralized, little or no pre-

precipitate occurs and the antitoxin is free in solution, whereas the toxin has disappeared.

Analysis of such solutions (Table III) shows that a rapid increase in non-protein nitrogen occurs as soon as the solution is brought back to pH 7.2. In the case of the toxin-antitoxin from crude antitoxin the increase amounts to nearly half the total protein nitrogen originally present while in the case of toxin-antitoxin from purified antitoxin the increase is much less. In both cases about one-third of the total antitoxin present is recovered free from toxin. This is similar to Pappenheimer's results with pepsin which appears to attack the antibody as well as the toxin. Since trypsin does not attack antitoxin alone nor toxin-antitoxin unless it is first acidified, the results indicate that the toxin-antitoxin complex is changed by acidification and that it is then hydrolyzed by trypsin in such a way as to destroy the toxin and also part of the antitoxin molecule.

If purified antibody, obtained by dissociating the toxin-antitoxin complex with acid alone (*cf.* Table II) is mixed with trypsin some of the protein is digested but there is also a loss of antitoxin. These results indicate that trypsin hydrolyzes the toxin-antitoxin complex in a different way from that in which it hydrolyzes either the toxin or antitoxin alone. The toxin-antitoxin complex acts in this respect like a distinct protein rather than a loose combination of toxin and antitoxin. This result confirms Heidelberger and Kendall's assumption that the toxin-antitoxin complex is a definite chemical compound. The reaction is peculiar in that a protein is left after the reaction is completed. This is also the case in the clotting of milk by either pepsin or chymotrypsin and also in the formation of pepsin, trypsin, or chymotrypsin from their precursors. All other proteolytic reactions, however, result in complete destruction of the protein.

Effect of Varying Conditions on the Amount of Antitoxin Recovered

The digestion of toxin-antitoxin just described is affected by the following variables: pH, time at each pH, concentration of salt, kind of salt, temperature, concentration of toxin-antitoxin, and concentration of trypsin. Obviously determination of strictly optimum conditions with such a large number of variables would involve an enormous number of experiments. Preliminary experiments showed that the time of standing in acid and also the pH of this acid solution made little difference so long as the toxin-antitoxin complex was dissolved (Table IV). Trypsin may be added before neutralization or immediately afterwards without affecting the yield. If the toxin-antitoxin complex is allowed to stand at pH 7.2 for several hours and trypsin then added, no antitoxin is recovered. The same results are obtained with toxin-antitoxin from either plasma or from purified antibody, except that in the latter case a small amount of antibody may be recovered without trypsin if the solution is allowed to stand for 20 hours or more in acid. The concentration of trypsin may be varied from 0.01 to 0.5 mg. per ml. without affecting the percentage of antibody recovered. The yield is the same with 0.5 M phosphate as with 0.05 M phosphate but if the solution is acidified with acetic acid and then neutralized with ammonia the yield is much less. The yield of antibody recovered without trypsin, however, is greater in acetic acid. The yield is the same at temperatures from 10–40°C. except that the reaction is slower at the lower temperatures.

The yield increases as the concentration of toxin-antitoxin decreases but below about 0.5 mg. protein nitrogen per ml. the increase in yield is small.

An outline of the method as finally worked out for handling large quantities of

TABLE IV

Yield of Antitoxin Recovered after Various Times of Standing at pH 3.7 and pH 7.4 with or without Trypsin

Antidiphtheria plasma mixed with equivalent volume of toxin, stood 24 hrs. 25°C., centrifuged. Precipitate washed two times with $N/10$ pH 7.4 phosphate. Suspended in $N/10$ pH 7.4 phosphate, volume equal to that of plasma. 25 ml. + 25 ml. $N/10$ hydrochloric acid (pH 3.7) 2 hrs. 5°C. as noted. 5 ml. + 1 ml. $N/2$ pH 7.4 phosphate. Stood at 25°C. 0.05 mg. crystalline trypsin nitrogen per ml. added as noted.

Toxin-antitoxin from antidiphtheria plasma				
Time at pH 3.7-4.0		Time at pH 7.2-7.6		Per cent original antitoxin recovered
hours		hours		
Without trypsin	With trypsin	Without trypsin	With trypsin	
0	0	0	24	0
0.01	0	0	4	25
0.0	0.01	0	4	30-50
1	0	0	4	30-50
0	1	0	4	50
7	0	0	4	35-50
0	7	0	4	50-70
24	0	0	4	35-50
0	24	0	4	50-70
0	24	0	24	50-70
1	0	0.15	24	25
1	0	0.50	24	30
1	0	4	24	0
1	0	24	24	0
24	0	24	0	10
Toxin-antitoxin from purified antibody				
0.01	0	0	24	50-60
0.5	0	0	24	50-60
20	0	0	24	50-60
20	0	24	0	15
0	0	4	4	<10

material is shown in Table V. All filtrations were carried out with suction using No. 3 Whatman paper and large Buchner funnels so that the final filter cake is not more than a few millimeters thick. If smaller filters are used the filtration may be slow.

Six different lots of antidiphtheria plasma and toxin have been used in the course of the present experiments. The results in general have been the same but the per cent of the original antitoxin recovered after treatment with trypsin varied from

20-60 per cent. It is not possible to state at present whether this variation is accidental or is caused by differences in the toxin or antitoxin preparation used.

The precipitate (3P) which forms after neutralization consists partly of unchanged toxin-antitoxin and a further 20-40 per cent antitoxin may be recovered by treating this precipitate as described for the original toxin-antitoxin suspension.

Filtrate No. 3 from this precipitate contains 20-60 per cent of the original antibody and has a titer of 300-400 L /mg. protein nitrogen. The protein in this solution is 90 per cent or more precipitated by toxin. The solubility of the protein in this preparation, however, varies markedly with the quantity of solid present; in fact, about as much as does the solubility of serum globulin.

The protein may be further fractionated by precipitation with ammonium sulfate (Table V).

Most of the antibody is soluble in 0.33 saturated ammonium sulfate but precipitates at 0.5 saturated ammonium sulfate, as found by Pappenheimer and by Pope for antibody prepared by pepsin digestion. The fraction soluble between 0.33 and 0.60 saturated ammonium sulfate has a titer of from 500-700 flocculation units milligram protein nitrogen, or by the animal protection test. (The writer is indebted to Dr. W. E. Bunney at E. R. Squibb and Sons, New Brunswick, N. J., for carrying out this determination.) It is strictly homogeneous in the ultracentrifuge with a sedimentation constant of 5.7×10^{-13} . The diffusion constant is 5.50×10^{-7} $\text{cm}^2/\text{sec}^{-1}$ and the molecular weight is 90,500 (Rothen, 1941). The material shows only one boundary in the electrophoresis cell at pH 7.3 or 3.0. (In dilute phosphate or veronal buffer there is some reversible spreading but measurements of electroendosmosis of this buffer in the microscopic cataphoresis cell (Northrop and Kunitz, 1925) show marked flow of water at the glass liquid interface. Addition of $M/20$ calcium chloride, as is to be expected, completely prevents the electroendosmosis and also abolishes the reversible spreading.) This fraction probably corresponds closely to the preparation obtained by Pappenheimer and by Pope.

The solubility of this fraction, however, still varies markedly with the amount of solid (Fig. 1, second curve) showing that more than one protein is present in spite of the fact that it is homogeneous by ultracentrifuge and electrophoresis. This result is simply another example of the fact that the solubility method, which is theoretically identical with the classical melting point method, will distinguish between closely related proteins which are indistinguishable by other methods. Thus Landsteiner and Heidelberger (1923) found that hemoglobin from donkey and horse could be distinguished by this method although serologically they are extremely similar. Crystalline pepsin (Herriott, Desreux, and Northrop, 1940) prepared without special purification, is strictly homogeneous by electrophoresis or ultracentrifugation but does not have constant solubility and may be shown to contain several different proteins. Mixtures of samples of chymotrypsinogen of different solubilities are indistinguishable by electrophoresis (Butler, 1940) as are mixtures of pepsin and iodinated pepsin (Herriott, 1941) although in the latter case the solubilities are very different. Egg albumin from closely related species is also indistinguishable by the electrophoresis technique although they may be distinguished by serological tests (Landsteiner, Longworth, and van der Scheer, 1938).

Since the solubility of the solid in a dilute suspension is different from that in a

TABLE V
Preparation of Pure Diphtheria Antitoxin

	No.	Vol.	PN ml. mg.	L_f ml.	Total L_f	$\frac{L_f}{\text{mg. PN}}$
33 liters diphtheria toxin + 4 liters antidiphtheria plasma, 25°C. 24 hrs.....	1	37 liters	3	60	2×10^6	20
Siphon off supernatant, precipitate suspension + 25 gm. Hyflo per liter, filter and wash precipitate 3 times with 1 liter M/10 pH 7.4 phosphate. Precipitate stirred + 5 liters 0.05 M KH_2PO_4 , titrate to pH 3.5, + 2.5 gm. crystalline trypsin. Stand 25°C. 24 hrs. Filter. Titrate filtrate to pH 7.4, cloudy.....	2	6.3 liters	0.3	100	6×10^5	350
No. 2 + 700 ml. saturated ammonium sulfate (0.1 saturated) + 60 gm. Hyflo, filter. Filtrate clear.....	3	6.8 liters	0.17	70	5×10^5	400
Precipitate	3P					
No. 3 + 2.3 liters saturated ammonium sulfate (0.33 saturated) + 50 gm. Hyflo. Filter. Precipitate (0.1-0.3 fraction).....	4				1×10^5	350
Filtrate.....	5	8.6 liters	0.10	$\begin{cases} 60 \\ 50 \end{cases}$	4×10^5	500
No. 5 + 2.1 liters saturated ammonium sulfate (0.45 saturated) 2 hrs. 25°C. + 50 gm. Hyflo. Filter. Precipitate (0.33-0.45 fraction).....	6				2×10^5	450
Filtrate.....	7	11 liters	0.03	20	2×10^5	700
No. 7 + 1500 gm. ammonium sulfate (0.65 saturated) 10°C. Settle. Decant supernatant. Precipitate suspension + 20 gm. Filter-cel. Filter. Precipitate.....	8				2×10^5	700
Precipitate 8, stir + 3 liters (0.5 saturated ammonium sulfate, 0.05 M PO_4 pH 7.4), filter. Filtrate.....	9	3 liters	0.07	$\begin{cases} 45 \\ 50 \end{cases}$	1.5×10^5	$\begin{cases} 650 \\ 700 \end{cases}$
3 liters No. 9 + 60 ml. (2.5 N H_2SO_4 , 0.5 saturated ammonium sulfate) 25°C. 1 hr. + 20 gm. Filter-cel. Filter. Filtrate.....	10	3 liters	0.05	$\begin{cases} 30 \\ 35 \end{cases}$	1×10^5	$\begin{cases} 600 \\ 700 \end{cases}$

TABLE V—*Concluded*

	No.	Vol.	PN ml. mg.	$\frac{L_f}{\text{ml.}}$	Total L_f	$\frac{L_f}{\text{mg. PN}}$
Titrate No. 10 to pH 6.8, stir in saturated ammonium sulfate slowly until silky precipitate forms (about 200 ml. saturated ammonium sulfate). Stand 6°C. 24 hrs. Decant and centrifuge. Precipitate suspension, needles, plates and some amorphous. Suspend in 50 ml. 0.5 saturated ammonium sulfate.	11	50 ml.	1.0	$\begin{Bmatrix} 650 \\ 700 \end{Bmatrix}$	3×10^4	$\begin{Bmatrix} 650 \\ 700 \end{Bmatrix}$
No. 11 + 10 ml. water (clear) + saturated ammonium sulfate till slightly turbid. Stand 25°C. 2 hrs., thin plates, no amorphous. Stand 25°C. 24 hrs., centrifuge. Precipitate + 20 ml. 0.5 saturated ammonium sulfate. Precipitate suspension	12	20 ml.	1.0	$\begin{Bmatrix} 600 \\ 700 \end{Bmatrix}$	1.2×10^4	$\begin{Bmatrix} 600 \\ 700 \end{Bmatrix}$
No. 12 + 5 ml. water + saturated ammonium sulfate slowly until slightly turbid. Stand 25°C. 1 hr., heavy precipitate thin plates. Centrifuge. Dissolve precipitate + 10 ml. water.	13	10 ml.	1.0	$\begin{Bmatrix} 650 \\ 700 \end{Bmatrix}$	6×10^3	$\begin{Bmatrix} 650 \\ 700 \end{Bmatrix}$

concentrated one, the composition of this solid must be different and hence a partial separation has been effected. On paper it is only necessary, therefore, to repeat the solubility experiment until a pure protein of constant solubility is obtained.² Actually, however, this cannot be done since the yield is small and the substance too unstable. The fraction soluble in 0.5 saturated ammonium sulfate but not at 0.65 has more nearly constant solubility, indicating that the more soluble fraction could be more easily purified than the less soluble. A very small amount of a protein having

² If the protein is a solid solution of two proteins and obeys Raoult's law then the maximum change in composition which can be obtained in one step is from F to CF when F is the original ratio of the two constituents and C is the ratio of their solubilities in the solvent used (Northrop and Kunitz, 1930). If a solvent could be found in which the solubility of one component was 0, then the separation would be complete in one step. For this reason it is to be expected that a better separation will be obtained in a solvent in which the total solubility is small and this is the case. A similar result was obtained in the case of pepsin (Herriott, Desreux, and Northrop, 1940).

constant solubility (bottom curve, Fig. 1) was eventually obtained by extracting with one-half saturated ammonium sulfate followed by precipitation at pH 3.5 as shown in the table. This protein is homogeneous by electrophoresis and ultracentrifuge and fulfills the criteria for a pure protein.

Inspection of the solubility curves shows that only sample 48B12 had strictly constant solubility. The other samples tested all showed minor discrepancies al-

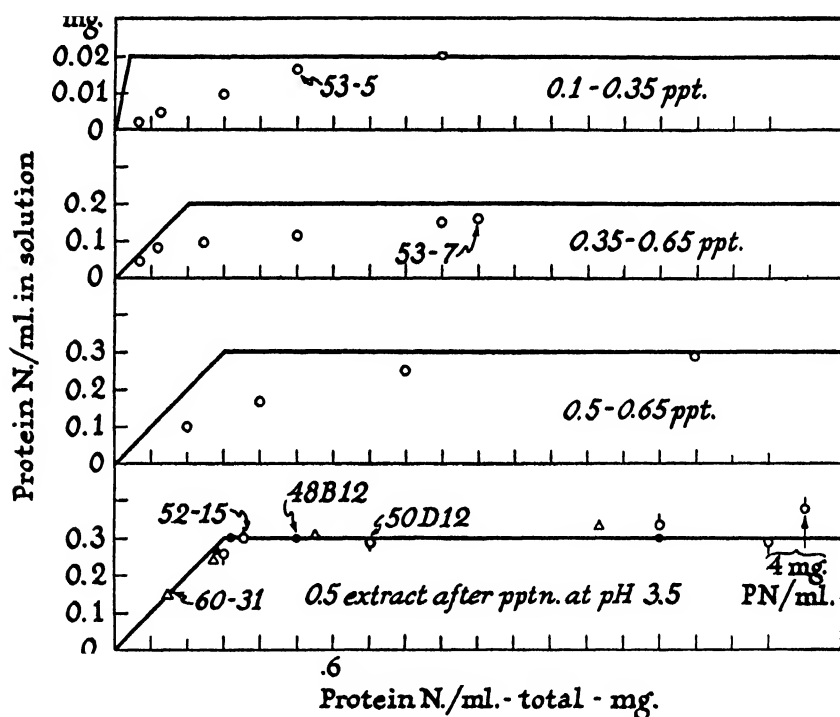


FIG. 1. Solubility curves of various antitoxin fractions. The protein nitrogen per milliliter in solution is plotted against the total protein nitrogen concentration. The solid lines are the theoretical curves for a solid phase of one component.

though the curves are better than those of other proteins with the exception of chymotrypsinogen, trypsin, and pepsin. The determinations were all made with the amorphous preparation since some decomposition occurs during crystallization and the crystals do not have as good a solubility curve. The samples tested in Fig. 2 were analyzed within a few hours after their preparation.

Solutions of the protein crystallize readily in the form of thin, more or less irregular plates (Fig. 2). These plates resemble very closely the first crystals of ribonuclease isolated by Kunitz (1940). On longer standing or on recrystallization the ribonuclease crystals become beautifully regular, whereas the antibody crystals remain about

the same.³ The first crystals which appear are the best and after a few hours crystallization stops. On recrystallization the same process is repeated. Crystals form rapidly and are as good as would be expected under the conditions but instead of improving on standing the crystallization again stops. The protein in the mother liquor cannot be crystallized and has a low solubility very much like that of the 0.5-0.6



FIG. 2. Crystals of diphtheria antitoxin.

fraction from which the pure protein was originally isolated (Fig. 3). At the same time, the time required for flocculation increases, when the antitoxin is mixed with

³ Large well formed prisms have appeared in two preparations after 3-4 months' standing. These preparations had been sealed with vaseline under a cover slip on a slide. The original poorly formed plates disappear as the larger crystals develop indicating that the large crystals are either the antibody itself or a decomposition product. It has not been possible so far to prepare enough of these crystals to test for antibody content.

toxin, although the final titer remains the same (Table VI). The results show that the pure protein is very unstable and is rapidly transformed to a slightly less soluble form. The presence of this form prevents crystallization. Most protein solutions, if allowed to stand in strong salt solution, form thin plates on the surface which may settle subsequently and which resemble somewhat the antitoxin plates. These surface films differ, however, in that they are more irregular and are usually spotted with

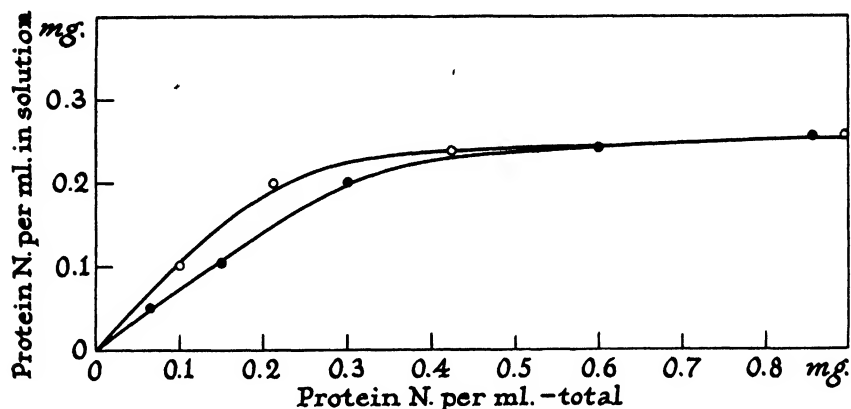


FIG. 3. Effect of standing in 0.4 saturated ammonium sulfate pH 7.0 on solubility of crystalline antitoxin in (0.5 saturated ammonium sulfate, 0.05 M pH 7.4 phosphate). Upper curve—solubility after 1 hour at 25°C. Lower curve—after 24 hours.

TABLE VI
Increase in Flocculation Time of Purified Antibody on Standing at 25°C.

1 ml. toxin (45 L_f /ml) +	0.2	0.25	0.3	0.4 ml. 87-1 (0.5 saturated ammonium sulfate extract)
Time at 25°C.	Time for flocculation 50°C.			
days	min.	min.	min.	min.
0		10	10	12
1	45	15-18	15-18	20
2	40	17	15	17
4		20	14	15

irregular markings. In addition the formation of the antitoxin plates is greatly accelerated by inoculation of the supersaturated solution while the formation of surface plates is not. Under these conditions crystallization may be practically complete in $\frac{1}{2}$ to 1 hour and it can be seen that the plates are formed in the body of the solution and not on the surface. The plates are faintly doubly refractile when observed with the Nicol prism and analyzer. An intense light is necessary as, owing to the thinness of the plates, the double refraction is slight. The straight lines in the photograph are not needles, but the edges of the plates. This is clearly evident under the microscope.

The plates are slightly less soluble than the amorphous form although the difference is much less than is the case with pepsin.

These results indicate that the plates from saturated solution of this protein are true crystals although final decision depends on the results of x-ray analysis.

TABLE VII

Per Cent Protein Nitrogen Precipitated from Various Samples of Antitoxin by Crude or Purified Toxin

5 ml. crude or purified toxin solution added to various samples of antitoxin containing equivalent number of units in N/10 pH 7.2 phosphate. Protein nitrogen per ml. determined. 50°C. 20 hrs. 25°C. 48 hrs. Centrifuged and protein nitrogen determined in supernatant (turbidity).

Sample No.	51A41	86-5	86B1	86-25	86A16	90-13
	Globulin fraction	0.1-0.3 sat. a.s. fraction	0-0.35 sat. a.s. fraction	0.4-0.5	crystals	(2 × ppt. + toxin)
<i>L_f</i> /mg. P.N.	150	500	250	800	750	900
Crude toxin						
Total P.N., mg.	2.25	0.75	1.1	0.60	0.60	0.48
P.N. in supernatant, mg.	1.95	0.06	0.12	0.03	0.06	0.07
Per cent P.N. in supernatant. . . .	86	8	10	5	10	14
Per cent P.N. in precipitate. . . .	14	92	90	95	90	86
Purified toxin (Pappenheimer)						
Antitoxin P.N., mg.	2.0	0.72		0.48	0.50	0.50
Toxin P.N., mg.	0.11	0.22		0.22	0.22	0.14
Total P.N., mg.	2.11	0.94		0.70	0.72	0.64
P.N. in supernatant, mg.	1.97	0.04		0.048	0.08	0.03
Per cent total P.N. in supernatant	93	4		7	11	4
Per cent P.N. in precipitate. . . .	7	96		93	89	96

Precipitation of Various Fractions of Purified Antitoxin by Crude and Purified Toxin

Mixtures of diphtheria toxin and antitoxin precipitate over a rather narrow range of concentrations when allowed to stand. Preliminary experiments showed that precipitation increased for 10 or 15 minutes at 50°C. but after 20 hours remained constant. The experiments shown in Table VII were made at the equivalent point; *i.e.*, with that proportion of toxin and antitoxin which first precipitates. The results with other mixtures near this range are not significantly different.

The first sample, 51A41, was a globulin fraction prepared directly from antidiph-

theria plasma without previous precipitation with toxin. It had an antitoxin titer of about 150 units per mg. protein nitrogen and only about 7 per cent of the total nitrogen was found in the precipitate. This figure, of course, is subject to a large error since it is determined by difference. The other preparations represent different fractions obtained by fractional precipitation with ammonium sulfate and have antitoxic titers varying from 250 units per mg. protein nitrogen to 900. However, as Table VII shows, 90 per cent or more of the total protein nitrogen is precipitated from all of these preparations. The table also shows that the amount of protein nitrogen left in the supernatant when crude toxin is used is about the same as when purified toxin is used. Evidently the protein in the crude toxin preparation used was practically all toxin protein.

It follows from these determinations that the various fractions must combine with different quantities of toxin. 0.72 mg. protein nitrogen of 86-5, which is the fraction obtained between 0.1-0.3 ammonium sulfate precipitated 0.22 mg. of toxin protein nitrogen, a ratio of about 3 to 1, whereas the preparations having a higher antitoxic titer precipitate an amount of toxin protein equivalent to about one-half the antitoxin. These figures are somewhat uncertain owing to the fact that some protein nitrogen is not precipitated and it is not possible to say whether this is antitoxin protein or toxin protein or partly both. The precipitate has no measurable solubility in $m/20$ phosphate buffer and it seems unlikely that the protein remaining in solution was really due to the solubility of the precipitate. Since the precipitate appears to be completely insoluble it follows that the quantity formed will be independent of the volume and this is the case as Heidelberger and Kendall (1935) have previously found. However, this would be true of any insoluble precipitate and it does not seem possible to draw any conclusions from this result concerning the composition of the precipitate.

Kekwick and Record (1941) have obtained partially purified antitoxic preparations by means of electrophoresis which also showed varied combining ratios with toxin and have suggested, therefore, that there are at least two antitoxins. The present experiments confirm this conclusion.

The Effect of the Purity of the Preparations on the Precipitation Zone

The precipitation of crude diphtheria toxin with antitoxin is peculiar in that the precipitation range is narrow. For this reason the titration is accurate to about ± 20 per cent. The precipitation of crude or purified toxin solutions with different dilutions of antidiphtheria plasma or purified antibody is shown in Table VIII. The table shows that with crude toxin and antidiphtheria plasma only one tube shows complete precipitation; *i.e.*, either twice as much plasma or one-half as much plasma does not give a flocculent precipitate. When crude toxin is precipitated by purified antibody the range of precipitation is doubled and it requires a larger excess of antibody to prevent precipitation. For this reason the titration of purified antitoxin is much less accurate than the titration of antidiphtheria plasma. When purified toxin is mixed with purified antibody the range is extended so that complete precipitation occurs even in the presence of an excess of antibody which is equivalent to four times the toxic equivalent and extends far down into the region of excess toxin. These and the preceding experiments indicate that a possible cause for the varying composition of

toxin-antitoxin complexes is due to the fact that there are more than one toxin and more than one antitoxin.

The purified toxin used in these experiments was kindly supplied by Dr. A. M. Pappenheimer, Jr. It was homogeneous by electrophoresis or ultracentrifuge but may still have contained more than one protein since its solubility has not been tested (Petermann and Pappenheimer, 1941).

TABLE VIII

Precipitation of Antidiphtheria Plasma or Purified Antibody with Crude or Purified Toxin

1 ml. toxin solution containing 40 L_f /ml. in $N/10$ pH 7.4 phosphate mixed with 1 ml. various antitoxin preparations diluted as noted with $N/10$ pH 7.4 phosphate and allowed to stand at 25°C.

Time at 25°C.	Dilution of plasma....	Amount of precipitation							
		1	1/2	1/4	1/8	1/16	1/32	1/64	1/168
<i>hrs.</i>		Crude toxin + crude antibody							
1.5		—	+	++	C	+	—	—	—
24		—	+	++	C	+	—	—	—
		Crude toxin + purified antibody 750 L_f /mg. P.N.							
1.0		—	—	++	C	+	—	—	—
24		—	+	C	C	+	—	—	—
		Purified toxin + purified antibody							
1.0		+	+++	C	C	+	—	—	—
24		+	C	C	C	C	C	C	+++

Effect of Salts on the Precipitation of Toxin-Antitoxin

The formation of toxin-antitoxin precipitates is similar in many respects to the specific agglutination of bacteria. In the case of bacterial agglutination it has been repeatedly shown that (Bordet, Joos, Bechold, Porges, Porges and Prantschoff, Northrop and DeKruif (*cf.* Topley and Wilson, page 154)), the reaction takes place in two steps. The first step is the combination of antibody with the bacterial cell. This is the specific step in the reaction and, as Heidelberger and Kendall's (1935) results indicate, is very likely a chemical reaction. In the absence of electrolytes and especially in slightly alkaline solution no agglutination takes place. However, if electrolytes are now added to such sensitized suspensions, agglutination occurs and the effect of the electrolytes may be predicted from the effect of electrolytes upon colloidal suspensions in general. Such suspensions are characterized by the fact that they are agglutinated by ions having an opposite charge from the particles and that the agglutinating concentration decreases rapidly as the valence of the ion increases. This observation was originally made by Hardy and applies to the oil droplet (Powis, 1924), collodion particles coated with denatured egg albumin, or denatured proteins

in general (Loeb, 1924), or sensitized bacteria (Northrop and DeKruif, 1922). This valency effect of salts is quite distinct from the effect of salts on solutions of crystalloids or on the solubility of native proteins. It appears to be characteristic of antigen-antibody complexes that the solubility or stability of the complex is determined by the electrical charges as is the stability of colloidal suspensions in general, while the solubility of the antigen or antibody separately may be affected by electrolytes in an entirely different way and in the same way as are solutions of crystalloids or native proteins.

If toxin-antitoxin complex is formed from antidiphtheria plasma and crude toxin and then washed repeatedly with dilute phosphate buffer it remains completely in-

TABLE IX

Precipitation of Solution of Toxin-Antitoxin in Water by Acid and Salts

Sample 142-5 (7.5 mg. P.N./ml.) diluted 1/20 with water-clear—No. 1.

2 ml. No. 1 +	0	0.1	0.2	0.3	0.5	0.7	1.0 ml. N/4,000 acetic acid.
Precipitation	—	+	+	++	C	C	+
pH (brom cresol purple)	7.6	6.6	6.55	6.5	6.45	6.4	6.3

1 ml. No. 1 + 1 ml. various salt solutions pH 7.4 in N/50 pH 7.4 veronal buffer; dilutions made with water.

Salt	Final concentration salt												
	M/20	M/40	M/80	M/160	M/320	M/640	M/1280	M/2000	M/5000	M/10,000	M/20,000	M/40,000	M/80,000
	Precipitation after 20 hrs. 25°C.												
NaCl.....	C	C	+	—	—								
pH 7.4 veronal.....	C	++	+	—	—								
Na ₂ SO ₄	C	C	++	+	—	—							
CaCl ₂	C	C	C	C	C	C	++	+	—				
LaCl ₃				C	C	C	C	C	C	C	C	C	++

soluble. If this precipitate is then washed repeatedly with distilled water it becomes gelatinous and finally dissolves. The solution in distilled water alone at about pH 7.0 is more or less opalescent but at pH 8.0–9.0 it is quite clear and resembles a solution of denatured protein. If electrolytes or acid are now added to such a solution precipitation occurs and the concentration of electrolytes required decreases rapidly with the valency of the positive ion (Table IX). The results in general are exactly similar to those obtained with the oil droplets, etc. described above. In addition, acid causes precipitation over a narrow range of pH of about 6.4–6.5, the width of this range depending upon the concentration of the solution.

As in the case of bacteria, therefore, the toxin-antitoxin complex behaves in regard to salt precipitation like a preparation of denatured serum protein as Shibley (1926) has emphasized in connection with bacterial agglutination. In the case of the toxin-antitoxin complex, however, the protein is probably not denatured since the results of the experiments with trypsin digestion previously described (Table I) show that this

complex is not attacked by trypsin, whereas if it is boiled the complex is rapidly digested. Trypsin attacks native proteins slowly if at all so that the failure of trypsin to digest the toxin-antitoxin complex indicates that the protein is still native.

Stability of Purified Antitoxin

The experiments described in connection with the crystalline antitoxin show that the strictly homogeneous protein is extremely unstable and changes very rapidly to a slightly less soluble form. After this preliminary change, however, the preparation is quite stable over a pH range of 2.0-8.5. Within this range little or no loss of ac-

TABLE X
Inactivation of Purified Diphtheria Antitoxin at Various pH

Purified antitoxin (0.4 saturated-0.5 saturated ammonium sulfate fraction) in $N/10$ pH 7.4 phosphate and 0.05 saturated ammonium sulfate; P.N./ml. 0.5 mg. 5 ml. plus noted ml. sodium hydroxide or sulfuric acid. Stand 25°C. 1 ml. sample + 4 ml. $N/2$ pH 7.4 phosphate and titrated against crude toxin.

Ml. 2 N sodium hydroxide	1.0	0.5	0.25	0.1	0	0.2	0.5	1.0	2.0
Ml. 1 N sulfuric acid. . . .	<1.0	<1.0	1.5	3.6	7.4	8.0	8.5	9.0	9.8
pH (colorimetric)									
Time at 25°C.	<i>L_f/ml. after standing at 25°C. as noted</i>								
<i>hrs.</i>									
0.25	$\begin{Bmatrix} 250 \\ 200 \end{Bmatrix}$	$\begin{Bmatrix} 350 \\ 300 \end{Bmatrix}$	400	400	400	400	400	$\begin{Bmatrix} 200 \\ 300 \end{Bmatrix}$	$\begin{Bmatrix} 200 \\ 150 \end{Bmatrix}$
24	50	100	200		$\begin{Bmatrix} 350 \\ 400 \end{Bmatrix}$	$\begin{Bmatrix} 350 \\ 400 \end{Bmatrix}$	$\begin{Bmatrix} 350 \\ 400 \end{Bmatrix}$	$\begin{Bmatrix} 200 \\ 250 \end{Bmatrix}$	150
Precipitation after 24 hrs.	+++	+++	+++	-	-	-	+	++	++

tivity is observed in 24 hours at 20°C. (Table X). In more alkaline or more acid solutions precipitation occurs and there is marked loss in antitoxic value.

Carbohydrate Content of the Antibody Preparations

Petermann and Pappenheimer (1941) have found that the carbohydrate content of purified antibody prepared by digestion of the toxin-antitoxin complex by pepsin is higher than in crude antibody fractions prepared directly from immune serum. The carbohydrate content of the antibody fraction prepared by the present method does not appear to differ significantly from that of antitoxin prepared by fractionation direct from antisera (Table XI). Some variation was found in the carbohydrate content and one preparation, 62-2, gave 3.4 per cent. This variation in the carbohydrate content and the general character of the preparations suggests the possibility that a part, at least, of this carbohydrate, is derived from the serum mucoid and is not a part of the antitoxin molecule itself. However, all attempts to reduce the carbohydrate content below about 2 per cent have been unsuccessful.

The Relation of the Purified Antibody to the Proteins of Normal Serum

It has frequently been suggested that antibodies are formed by a slight modification of normal serum proteins. They are, however, extremely closely related to them as evidenced by the fact that some of Pappenheimer's preparations, which contain less than one-half antibody, were homogeneous by electrophoresis and ultracentrifuge,

TABLE XI
Glucose Content of Various Antitoxin Preparations

Per cent glucose analyzed according to Sørensen and Haugaard (1933) compared with standard glucose solutions					
Sample.....	51A41 0.4-0.5 frac- tion from antidiph- theria plasma	86-25 0.4-0.5 sat- urated ammo- nium sul- fate frac- tion from purified antitoxin	86A67 Purified toxin once crystal- lized	62-2 Purified toxin two times crystal- lized	90-15 Antitoxin precipi- tated two times with toxin
Per cent glucose.....	2.4	2.0 2.4	2.8	3.4	2.8

TABLE XII
Fractional Precipitation of Antidiphtheria Plasma or Normal Plasma and Pure Antitoxin with Varying Concentrations of Ammonium Sulfate

Ammonium sulfate concentration	Antidiphtheria plasma			Normal plasma + purified antitoxin			Normal plasma
	Per cent P.N.	Per cent L_f	L_f /P.N.	Per cent P.N.	Per cent L_f	L_f /P.N.	Per cent P.N.
<i>per cent saturated</i>							
Original	(100)	(100)	30	(100)	(100)	10	
0-35	44	10	10	45	<10	<10	40
35-45	19	50	75	8	50	60	10
45-55	9	40	100	6	30	50	10
55-65	25	<10		30	<10		40

and also by the fact that the antibody can only be separated from the normal proteins by fractional precipitation with extreme difficulty.

If the purified antibody obtained by the action of trypsin were different from the form in which it occurs in the original immune plasma, it might be expected that the purified antibody could be separated from normal plasma more easily than the naturally occurring antibody can be separated from the proteins of immune plasma. In order to test this assumption antidiphtheria plasma containing about 500 antitoxic units per ml. and an "artificial" immune plasma containing the same number of anti-

toxic units prepared by adding purified antitoxin to normal plasma, were precipitated by varying concentrations of ammonium sulfate. The results of the experiment are shown in Table XII. It is evident that the antitoxin distributes itself in the same way in both cases since the percentage of the original antitoxin found in the various fractions is within the limits of error the same. The antitoxic titer per milligram of protein nitrogen of the various fractions is also about the same and it is just as difficult to concentrate the antitoxin from the "artificial" immune plasma as from natural immune plasma.

Immunological Relations of the Purified Antitoxin to Normal Serum

Some of the immunological properties of the purified antitoxin have been determined by Dr. Carl TenBroeck. The serum of a rabbit immunized against normal horse serum gave a precipitate with 1/4,000 ml. normal horse serum (containing about 0.002 mg. protein nitrogen) but gave no precipitate with 1 ml. of a solution of purified antibody containing 1/10 mg. protein nitrogen.

Guinea pigs sensitized by the subcutaneous injection of 0.003 mg. of purified antibody protein gave a typical anaphylactic reaction 3 weeks later when 0.05 mg. of antibody protein nitrogen was injected intravenously. Similarly sensitized guinea pigs failed to react to normal horse plasma diluted 1:10, but 4 out of 6 reacted to the normal undiluted plasma containing 0.5 mg. of protein nitrogen.

The purified antitoxin is, therefore, antigenically distinct from the normal serum proteins. The fact that guinea pigs sensitized with purified antibody react with large amounts of normal horse serum probably indicates the presence of minute amounts of normal protein in the purified antibody preparation. Extremely minute amounts are sufficient to sensitize an animal and it is quite possible that such minute amounts of normal protein could be carried through the various steps of purification.

Experimental Methods

Protein Nitrogen.—1 ml. sample containing from 0.01–0.10 mg. protein nitrogen was added to 9 ml. (2.5 per cent trichloroacetic acid, 10 per cent saturated ammonium sulfate) and boiled. The suspension was allowed to cool and the turbidity measured against a standard suspension of purified antibody in a Klett photoelectric colorimeter. The standard suspension was analyzed by micro-Kjeldahl. The values for protein nitrogen in the purified samples are accurate to ± 5 per cent but those of the crude preparations and especially of the plasma fraction may be in error by 20–30 per cent since the turbidity factor varies with different proteins.

Flocculation Test.—Varying quantities of the solution to be tested were added to 1 ml. of toxin solution which had been titrated against a standard antibody preparation. The tubes were immersed in a 50°C. water bath and the end-point taken as that tube which first showed a flocculent precipitant. With antidiphtheria plasma this method is accurate to ± 20 per cent but with purified antibody the precipitation range is much wider and the error may be ± 20 –30 per cent.

Solubility Determination.—50–100 mg. of the sample were precipitated by 0.5 saturated ammonium sulfate, centrifuged, and the precipitate stirred with 5 ml. (0.5 saturated ammonium sulfate, 0.05 M pH 7.4 phosphate buffer) and centrifuged.

This process was repeated until the protein nitrogen per ml. of the supernatant was the same in two successive supernatants. The solid is now in equilibrium with this solution (*cf.* Butler, 1940). 0.1, 0.2, 0.5, 1.0, and 2.0 ml. of the suspension are placed in small centrifuge tubes and the volume of each made up to 3 ml. with the above solvent. The tubes are allowed to stand with occasional stirring for 2 hours. 0.1 ml. samples are removed for determination of total protein nitrogen, the tubes centrifuged, and the supernatant analyzed for protein nitrogen. It is possible to make a complete solubility curve with 6.0–10 mg. protein nitrogen and all of this may be recovered except for the amount actually used in the analytical samples.

The analyses reported were carried out by Miss Elizabeth Shears.

SUMMARY

Purified preparations of diphtheria antitoxin have been obtained by digestion of the toxin-antitoxin complex with trypsin, followed by fractional precipitation with ammonium sulfate. The various fractions obtained in this way are all 90 per cent or more precipitated by diphtheria toxin but combine with different quantities of the toxin.

The fraction precipitated between 0.33 and 0.5 saturated ammonium sulfate is homogeneous by electrophoresis and ultracentrifuge but does not have constant solubility.

A small amount of a more soluble fraction has been obtained which does have constant solubility and satisfies the criteria of a pure protein. This protein crystallizes readily in poorly formed thin plates. It is very unstable and reverts to a less soluble non-crystallizable form. It has a sedimentation constant of 5.7×10^{-13} and a molecular weight of 90,500. It has an antitoxic value of 700–900 flocculation units per mg. protein nitrogen and has an antitoxic value by the protection test of about 700 units per mg. protein nitrogen.

The precipitation range of the purified antitoxin with purified toxin is much wider than that with crude preparations.

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THE PRESERVATION OF PNEUMOCOCCUS BY FREEZING AND DRYING

By ERNEST G. STILLMAN

(From the Hospital of The Rockefeller Institute for Medical Research)

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Swift (1937) has shown that when streptococci are completely dried while in the frozen state, they will survive unchanged for many years. Since pneumococci are more sensitive to chemical changes in their environment and undergo spontaneous autolysis more readily than do streptococci, it was of interest to determine how long pneumococcal cells would remain viable when kept in the desiccated state. In order to ascertain whether this method of preservation, which has proved so practical in the case of other bacteria, is equally applicable to pneumococci, the following experiments were carried out. During the winter of 1935-1936 freshly isolated strains of types I, II, III and VIII pneumococci were prepared and dried by the technic described by Swift. The viability of the bacterial cells preserved under these conditions was tested by cultural methods after a period of three years. The biochemical and immunological characteristics, as well as the virulence of the recovered cultures, were compared with those of the parent strain.

METHODS

Strains of pneumococcus of types I, II, III, and VIII freshly isolated from human sources and of known virulence were grown in flasks containing 50 ml. of blood broth for 18 hours at 37°. The cultures were centrifuged and the cells resuspended in 1 ml. of plain broth. Two-tenths ml. portions of the concentrated bacterial suspension were distributed in small glass tubes measuring $9\frac{1}{2}$ x 90 mm.

A slightly modified CO₂ method of freezing the cultures was

used. Instead of placing the CO₂ ice in the glycerol, the concentrated cultures were first rapidly frozen by holding them on a cake of CO₂ ice and then placed immediately in the already chilled glycerol in a desiccating jar. A large flat dish, containing P₂O₅, was placed on top of the tubes, and the cover of the jar was replaced and pressed down to insure a tight seal. The jar was exhausted of air by means of a vacuum pump. The desiccator was then placed in the refrigerating box overnight. A refrigerator box large enough to hold the desiccating jar, such as used by Swift, is cooled by placing 200 to 400 grams of solid CO₂ in the bottom. The exact amount needed to keep the inside of the apparatus slightly below 0°C. until desiccation is complete must be learned by experience. Upon removal from the desiccator, the tubes were immediately sealed by allowing heated sealing wax to run into the space above the cotton plug. Air bubbles, entrapped in the seal, can be released by gently re-heating the wax and rotating the tubes.

After the specimens had been stored for three years, single tubes were selected at random and opened. One ml. of blood broth was then added to each specimen and the mixture was incubated at 37°C. overnight. If growth was apparent the next day, the culture was plated on fresh blood agar. If no growth was apparent, a second tube of blood broth was inoculated with the contents of the first tube.

Since the presence of the minute traces of moisture will apparently allow autolytic enzymes to act and bring about lysis of the cells, the greatest care must be taken in sealing the tubes. After the tubes have been stored for a few months, gross defects of the seal can be detected by gummy appearance of the contents. However, the physical appearance of the dry contents is not necessarily an index of the viability of the organisms. In order to determine how accurately viability could be foretold by the gross appearance of the material in the tube, a note of the general condition of a number of specimens was made before the tubes were unsealed and tested. Of 125 tubes containing dried type I organisms which appeared in good condition, only 71 or 54 per cent yielded growth when subsequently cultivated in blood

broth. Likewise, of 114 apparently well-preserved specimens of type II pneumococci, only 89 or 78 per cent showed the presence of living organisms. From this it is evident that little can be foretold by the gross appearance of the dried material.

EXPERIMENTAL

Although originally 5 dried specimens of each strain were prepared, in the course of 3 years a number of them had been used, so that at the end of this period there remained only 1 or 2 tubes of certain cultures. In the majority of instances, however, 4 or 5 specimens of each strain were still available.

In table 1 are given the results of culturing 772 specimens of the frozen and dried pneumococci. From the data presented,

TABLE 1
Incidence of recovery of cultures of pneumococci after preservation for 3 years

TYPE OF PNEUMOCOCCUS	NUMBER OF SPECIMENS	VIABLE	PER CENT VIABLE
I	298	127	42
II	191	132	69
III	168	105	62
VIII	115	83	72
Total	772	447	57

it is seen that 57 per cent of the specimens that had been stored for 3 years still showed the presence of viable pneumococci. The number of viable specimens varied with the different types. Of the 298 samples of dried type I pneumococci, only 42 per cent yielded growth when transplanted in suitable medium. Sixty-nine per cent of the 191 specimens of dried type II pneumococci and 62 per cent of the 168 tubes containing dried type III cells showed the presence of viable organisms. From these results it would seem that the capsular development bore no relationship to the length of time an organism would live. This is further exemplified in the case of the type VIII pneumococci which, although their capsules are smaller than those of Type III, were recovered in 72 per cent of the specimens tested.

In table 2 is shown the number of strains of pneumococci of various types which were recovered from frozen and dried preparations. From this it is seen that one or more cultures grew from only 61 per cent of the 68 strains of type I pneumococci. Forty-two of the 50 strains type II (84 per cent) and 43 out of 52 type III strains (82 per cent) were recovered. In the case of the type VIII pneumococci, of the 24 strains frozen and dried, 22, or 91 per cent, of the strains were recovered. From this it is clear that there is a difference in the average length of time for which different types of pneumococci will survive after freezing and drying.

TABLE 2
Viability of strains of pneumococci after 3 years

TYPE OF PNEUMOCOCCUS	NUMBER OF STRAINS	STRAINS RECOVERED	PER CENT
I	68	42	61.7
II	50	42	84
III	52	43	82.7
VIII	24	22	91
	194	149	76.8

As each set of tubes of the same strain was dried on the same day under identical conditions, the irregularity in the survival of pneumococci of the same strain is difficult to explain. Although all tubes were carefully sealed, it is possible that this irregularity may be due to failure of the sealing of individual tubes.

The presence of type-specific substances in these cultures was tested by agglutination in dilutions of pneumococcus sera. In no instance was any change or loss of specificity apparent. The virulence for white mice following injection was also tested. The organisms had lost none of their virulence during the 3 years in the dried state.

DISCUSSION

From these experiments it is evident that pneumococci may remain viable for as long as three years after being rapidly frozen and dried. It is essential that no moisture be permitted

to enter the tube. Tubes, which have been imperfectly sealed, may be easily detected by the gummy appearance of the material. But even tubes, the contents of which appear to be dry, fail to contain viable organisms. This is probably due to the penetration of minute amounts of moisture which allow the lytic enzymes to function. But there also may be a difference in the capacity of individual organisms from different strains to live. In the case of dried sputum (Stillman, 1938), where no attempt was made to exclude moisture, type I organisms lived on an average for 4 weeks and type III for 8 weeks.

When comparatively large quantities of organisms were dried in rabbit's blood and likewise stored without any attempt to exclude moisture (Stillman, 1940), although the period of survival was much shorter, the same tendency for the type I organisms to die most rapidly and the type III to live longest was also noted. In these frozen and dried specimens the type I cultures also apparently died more rapidly.

SUMMARY

1. Pneumococci in the dried state may remain viable for at least 3 years.
2. Variations in the viability in the different types of pneumococci have been observed under these conditions.
3. The serological specificity and virulence of strains recovered after freezing and drying remain unaltered.

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THE EFFECT OF SULFAPYRIDINE UPON THE DEVELOPMENT OF IMMUNITY TO PNEUMOCOCCUS IN RABBITS

By EDWARD C. CURNEN, M.D., AND COLIN M. MACLEOD, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, October 21, 1941)

Although the antibacterial action of sulfonamide compounds upon certain pathogenic microorganisms is now generally recognized, the effect of these drugs upon the immunological response of the animal or human host has not been completely elucidated. In view of the value of sulfonamides in the therapy of pneumonia and other diseases caused by pneumococci and because of the essential contribution of immunity to recovery from such infections, it seemed desirable to investigate further the action of sulfapyridine upon the immune processes.

In response to the antigenic stimulus of pneumococcus the tissues of man and certain animals are capable of elaborating type specific antibodies which circulate in the blood and can be determined by the appropriate techniques. In experimental animals it is also possible to recognize the occurrence of active immunity as indicated by increased resistance to homologous infection which may be present before and may persist after the period when circulating antibodies are detectable. In the process of spontaneous recovery from pneumococcal infection disposal of the invading microorganisms by the phagocytic cells cannot be accomplished without the development of a specific immune response.

The sulfonamide compounds exert a direct bacteriostatic effect upon various microorganisms. For these drugs to be effective therapeutically, however, the participation of specific humoral and cellular reactions of the host are essential requirements. The question of whether, in addition to their direct actions upon the bacteria, these compounds may not also exert an effect upon the defense mechanism of a host has been subjected to both experimental and clinical investigation. It has been demonstrated in mice injected intraperitoneally with pneumococci that the sulfonamide drugs do not affect the mobilization or activity of the phagocytic cells (1, 4). Although no evidence has been produced to show that these drugs stimulate or accelerate the process of antibody formation, the assumption that they exert no effect on the immune mechanism has not been unreservedly accepted.

In 1937 Buttle (2) observed that mice after recovery from infection with *Pneumococcus* Type I following treatment with the benzylidene Schiff's Base of 4:4'

diaminodiphenylsulfone were immune to reinfection with organisms of the homologous type. In the following year Whitby (3) confirmed this observation in mice treated with sulfapyridine. McIntosh and Whitby (4) found that at the end of 1 week mice which had survived 10,000 lethal doses of *Pneumococcus* Type I after oral treatment with sulfapyridine were completely immune to reinfection with a million fatal doses of pneumococci of the homologous type. This immunity was related quantitatively to the infective dose and was type specific. An inoculum of 1 million heat-killed pneumococci stimulated in vaccinated mice a degree of immunity comparable to that induced in mice following recovery under drug therapy from an otherwise fatal infection with 10,000 living pneumococci. In the latter instance the surviving animals on reinoculation were immune to an amount of virulent culture 100-fold greater than that used in the primary infection. The active immunity induced by vaccination or resulting from infection was demonstrable within 3 days. By the 4th or 5th day immunity was usually solid, and on the latter day the sera conferred passive protection on other mice. In the sera of mice immunized with *Eberthella typhosa* agglutinins were demonstrable by the 3rd day and increased in titer between the 4th and 7th days irrespective of whether or not sulfanilamide had been administered. These authors concluded that "the administration of sulphonamide drugs has no stimulating action on the body defenses, nor does such administration affect the quality, quantity, or speed of production of recognized specific antibodies." Experimental confirmation of Whitby's observation was reported in 1939 by Callerio (5) and MacLeod (6). MacLeod compared the survival rate following sulfapyridine therapy with the immune response in untreated animals, employing *Pneumococcus* Type I, a good antigen, and *Pneumococcus* Type III, a relatively poor antigen. He found that in mice infected with 10^{-2} cc. of a virulent culture of *Pneumococcus* Type I and treated with sulfapyridine, 95 to 100 per cent survived, whereas in mice similarly treated but infected with Type III pneumococci less than 10 per cent survived. In two groups of untreated mice immunized respectively with comparable doses of heat-killed pneumococci of Types I and III and infected 5 days later with 1,000 fatal doses of a virulent culture of the corresponding type, 95 to 100 per cent of those in the Type I group were immune while none of the Type III mice survived. An apparent correlation was observed not only between the survival rate and the relative antigenicity of the respective types of pneumococci, but also between the differences in the time required for the development of antibodies and the length of time during which it was necessary to continue sulfapyridine treatment. Larson, Bieter, and Levine (7, 8) demonstrated that rabbits which recovered from intradermal infection with *Pneumococcus* Type II following treatment with sulfapyridine were highly resistant to reinfection with the homologous type of organism 30 or more days later.

In the sera of patients with pneumococcal pneumonia the appearance of type specific antibodies at or about the time of spontaneous recovery is a well recognized phenomenon. However, in patients treated with sulfonamide drugs the appearance of these antibodies does not necessarily coincide with the defervescence frequently observed early in the course of illness.

Edwards, Kircher, and Thompson (9) tested 26 pneumonia patients treated with sulfapyridine for the development of dermal reactions to homologous type specific capsular polysaccharide as well as for the presence in their sera of type specific agglutinins. They concluded from the results of their observations that the immune responses which occur naturally in the course of untreated pneumonia are not affected by drug therapy.

Wood and Long (10) demonstrated specific protective antibodies in the sera of 10 out of 12 patients with pneumococcal pneumonia who were treated with sulfapyridine. They concluded that the antibody response in patients treated with sulfapyridine is similar to that observed in untreated patients.

Finland, Spring, and Lowell (11) studied the bactericidal power of whole blood as well as the development of homologous type specific agglutinins and mouse protective antibodies in the sera of a large number of patients with pneumococcal pneumonia treated with sulfapyridine. These investigators reported that mouse protective antibodies and agglutinins were rarely demonstrable in patients' blood before the 6th or 7th day although defervescence frequently occurred earlier. They concluded that the antibody response in patients treated with sulfapyridine was comparable in all respects to that observed in spontaneous recovery.

Fox, Rosi, and Winters (12) determined the time of appearance of specific antibodies in the sera of 50 adult patients treated with sulfapyridine. In all patients with proven pneumococcal pneumonia type specific agglutinins were demonstrable in the blood at some time in the course of illness. These same authors reported subsequently (13) that in a larger series 90 per cent of the patients developed type specific agglutinins by the end of the 2nd week of illness and that 50 per cent of those tested gave true positive skin reactions to the homologous type specific polysaccharide.

Knceland and Mullikin (14) tested the sera of 19 patients with pneumonia who were treated with sulfapyridine. They observed that positive precipitin reactions to the homologous capsular polysaccharide appeared in the sera of only four of their patients upon recovery. In a subsequent paper these authors (15) reported that type specific precipitins were detectable in the sera of only 8 of 30 sulfapyridine-treated patients as compared with 16 of 21 patients treated with sulfathiazole. They suggested that sulfapyridine by its action upon the invading microorganism diminishes the stimulus to antibody formation, whereas sulfathiazole, which they considered a less powerful bactericidal agent, exerts this effect to a lesser degree.

Bukantz and de Gara (16) demonstrated the presence of homologous type specific precipitins during recovery in the sera of nineteen of sixty patients with pneumonia who were treated with sulfapyridine.

In the animal experiments thus far reported, with the exception of those of McIntosh and Whitby, tests for resistance to homologous reinfection have been made at a time when immunity had reached or passed the peak of maximal response. In clinical studies the detection of circulating type specific antibody is necessarily limited by the methods which can be employed. The free circulating antibodies detectable *in vitro* by serological techniques probably repre-

sent an excess over the amount actually required for the initiation of recovery, except in those instances in which a severe infection overwhelms the total defense reactions of the patient. The results obtained by serological methods represent the summation of so many interrelated factors that any direct effect of sulfonamide therapy upon the specific immune response is necessarily obscured.

The present study was undertaken in order to determine whether or not the administration of a sulfonamide drug has a direct effect upon the development of immunity and to compare the relative sensitivity of various techniques used in measuring immunity, such as tests of active resistance to reinfection and the serological methods for determining the presence of circulating type specific antibodies. In order to standardize conditions as much as possible it seemed advisable to concentrate attention upon the immune response to a potent antigenic stimulus during the early stages. For this purpose *Pneumococcus* Type I was selected as the test organism, the rabbit as a suitable animal host, and sulfapyridine as a representative sulfonamide drug. These experimental conditions provided an opportunity to employ a highly virulent organism with potent antigenic capacity, an animal at once susceptible to infection with the organism and readily immunized by it, and a chemotherapeutic agent highly effective against the organism while not excessively toxic for the host.

EXPERIMENTAL

Rabbits were given intravenously a single immunizing injection of heat-killed *Pneumococcus* Type I. Approximately half of the vaccinated animals received sulfapyridine. Administration of the drug was started prior to vaccination, and was continued during the period when immunity was developing. The drug was discontinued before testing for active immunity by intradermal inoculation with *Pneumococcus* Type I.

Comparable groups of rabbits which had and which had not received sulfapyridine were injected intradermally with virulent *Pneumococcus* Type I, 48, 72, and 96 hours after vaccination. The immunizing injection was given to the respective series of animals on successive days so that all of the vaccinated rabbits in each experiment could be infected simultaneously.

The degree of immune response which had developed 48, 72, and 96 hours after vaccination was estimated in the sulfapyridine-"treated" and "untreated" rabbits¹ on the basis of their resistance to intradermal infection as well as by the presence

¹ For convenience the terms "treated" and "untreated" will hereafter be used to designate respectively those rabbits which received sulfapyridine and those to which no drug was given. It should be emphasized, however, that in the "treated" animals administration of sulfapyridine was in each instance discontinued 24 to 36 hours prior to infection and that none of the rabbits received chemotherapy following the intradermal inoculation.

in their sera of circulating type specific precipitins, agglutinins and mouse protective antibodies. For these determinations blood was drawn from each rabbit prior to vaccination and at appropriate intervals thereafter up to the time of intradermal infection.

Culture.—A virulent strain of *Pneumococcus* Type I (S V-I) was used both for preparing the vaccines and for intradermal injection. The virulence of this strain was enhanced and maintained by frequent passage in normal rabbits so that 0.2 cc. of a 1/500,000 dilution produced a fatal infection when injected intradermally.

Preparation of Vaccines.—The organisms from an 8 to 10 hour plain broth culture of *Pneumococcus* Type I were collected by centrifugation and resuspended in physiological salt solution equivalent in volume to 1/10 that of the original culture. The organisms were immediately killed by heating the bacterial suspension at 60°C. for 30 minutes in a water bath and then cultured to control sterility. A freshly prepared vaccine was used in each experiment. During the 48 hour period of use the vaccines were stored in the ice box and in all instances the bacterial cells retained their Gram-positive character.

Immunization.—"Chinchilla" rabbits weighing in the neighborhood of 2,000 gm., with a maximum variation of 1,560 to 2,500 gm. were kept under observation for a period of at least one week before use. Immunization was carried out by giving each rabbit a single intravenous injection of 1.0 cc. of vaccine corresponding to 10.0 cc. of original culture.

Sulfapyridine Administration.—Tablets of sulfapyridine (0.5 gm. Merck) were triturated in a mortar with 0.75 per cent solution of gum tragacanth so that 4.0 cc. of the resulting suspension contained 0.5 gm. of sulfapyridine. The drug was administered by stomach tube in doses of 0.5 gm., the 4.0 cc. of suspension being followed immediately by an equal volume of water to wash out the tube and insure passage of the whole amount into the stomach. Administration was begun prior to immunization, continued at intervals of 8 to 12 hours for approximately three days, and discontinued 24 to 36 hours before infecting the animals intradermally. The total dose of sulfapyridine given to each animal was 4.5 gm. with the exception of four rabbits which received reduced dosage because of toxic symptoms. Blood for sulfapyridine determinations was drawn at daily intervals during and after the course of drug administration until just prior to intradermal infection. The determinations were made according to the technique of Bratton and Marshall (17) employing a photoelectric colorimeter.

Determination of Type Specific Immunity by Means of Intradermal Infection.—At intervals of 48, 72, and 96 hours after injection of immunizing antigen, both the sulfapyridine treated and untreated groups of rabbits together with an adequate number of normal control animals were infected intradermally with a virulent culture of *Pneumococcus* Type I according to the method of Goodner (18). The infective dose, 0.2 cc. of a 1:5,000 dilution of an 8 to 10 hour blood broth culture, was invariably fatal for the controls.

The degree of immunity was estimated by comparing the clinical course of the experimental intradermal infection in the immunized and control animals. The observations included rectal temperatures twice daily, the severity and progression of the dermal lesions, and the results of quantitative cultures of the blood obtained

daily from the marginal ear veins. Animals which died were examined at autopsy and cultures were made of the heart's blood.

Determination of Circulating Antibodies. Precipitins.—Precipitin tests were carried out using 0.3 cc. respectively of undiluted rabbit serum and a 1:25,000 dilution of the acetylated pneumococcus Type I capsular polysaccharide. After thorough mixing of the contents, the tubes were incubated at 37°C. for 2 hours, refrigerated overnight, and read the following morning.

Agglutinins.—Tests for type specific agglutinins were carried out using serial dilutions of each serum. Formalin-killed cells of *Pneumococcus* Type I, suspended in a volume of saline equal to that of the original culture were employed. To 0.3 cc. of the serum dilutions, 0.3 cc. of antigen was added and after thorough mixing the tubes were incubated at 37°C., refrigerated overnight and read the following morning.

Mouse Protective Antibodies.—The sera of rabbits before immunization and at intervals of 48, 72, or 96 hours thereafter were tested in mice for protective antibodies by titrating dilutions of culture against a constant amount of serum.

The cultures of *Pneumococcus* Type I (S V-I) used in protection tests were of such virulence that the intraperitoneal injection of 10^{-8} cc. invariably proved fatal. The protection test was considered negative if 0.2 cc. of serum failed to protect 2 of 3 mice against 10^{-6} cc. of culture. Sera of rabbits which afforded protection to mice infected with 10^{-6} cc. (100 M.L.D.) of culture were also tested against 10^{-5} cc. (1000 M.L.D.). Amounts of culture greater than this were not used.

Blood Levels of Free and Acetylated Sulfapyridine.—Blood drawn at intervals during the period of sulfapyridine administration indicated satisfactory absorption of the drug. The blood levels of free and acetylated sulfapyridine, however, varied greatly in different animals under the same conditions. During the period of administration the highest concentration of free drug noted was 15.0 mg. per cent, the lowest 0.5 mg. per cent. The highest concentration of the acetylated compound was 71.2 mg. per cent, the lowest 4.3 mg. per cent. Although the blood level of free sulfapyridine dropped fairly rapidly in the interval between doses and after administration was discontinued, the acetylated form was eliminated much more slowly. In order that there should be little or no sulfapyridine remaining in the blood at the time of intradermal infection it was found necessary to discontinue administration of the drug 24 to 36 hours prior to infection. Determinations made 24 hours before intradermal infection showed that only 9 of the 34 animals still had free circulating sulfapyridine, although at this time the blood of all of the 22 rabbits tested for the acetylated compound showed its presence in amounts ranging from 1.3 to 71.2 mg. per cent.

Determinations made immediately before infection upon the blood of 30 immunized rabbits which received sulfapyridine showed the presence of the free drug in 3 instances. The blood of only one of these animals contained a measurable quantity (1.6 mg. per cent). On the other hand, the acetylated

compound was present in the blood of 11 out of 26 rabbits immediately before infection. The levels varied between 0.5 mg. and 5.7 mg. per cent. That these residual amounts of sulfapyridine remaining in the blood at the time of intradermal infection exerted no significant mitigating effect upon the course of the disease is indicated by the following observations. Sulfapyridine was present at the time of intradermal infection in the blood of three of the four treated animals which died. Furthermore, the fatal course of infection in four sulfapyridine treated rabbits which were not immunized, was essentially the same as that in the normal controls. Crystals of acetylated sulfapyridine were found at autopsy in the bladder and renal pelves of two rabbits which developed overwhelming bacteremia.

Toxic Effects of Sulfapyridine.—Almost all of the rabbits which received sulfapyridine showed some evidence of intoxication during the course of drug administration. This was manifested by varying degrees of anorexia, weakness, and loss of weight. Sulfapyridine intoxication may have been a contributory factor in the death of one rabbit which was infected 72 hours after immunization and died on the 4th day after a mild illness unaccompanied by bacteremia. Postmortem examination revealed a loss of 300 gm. in weight, a minimal skin lesion showing evidence of healing and no gross abnormalities of the organs. Analysis of the bladder urine disclosed the presence of acetylated sulfapyridine in a concentration of 2.5 mg. per cent.

It seems evident that the amount of drug used in these experiments was about the maximum that could be tolerated by the animals over the period of administration.

Effects of Intravenous Injection of Pneumococcal Vaccines.—The intravenous injection of heat-killed *Pneumococcus* Type I was frequently followed by an immediate but transient elevation of temperature. No other untoward reactions were noted. The variations of the immune response in sulfapyridine treated and untreated animals at different intervals following immunization are considered later in detail.

Characteristics of the Disease Produced by Intradermal Infection.—The disease produced in rabbits by the intradermal injection of *Pneumococcus* Type I has been thoroughly described by Goodner (18, 19). The course of the illness in the 26 control animals corresponded closely to his description. The disease was characterized by high sustained fever, extensive dermal lesions, progressive bacteremia, and an invariably fatal termination within 6 days. In immunized rabbits modifications of these findings were progressively more in evidence as the interval between immunization and infection was increased. As a basis for the estimation of active immunity, the effects of infection at different intervals after immunization in rabbits which received sulfapyridine are compared with the reactions of animals to which no drug was given. These data are presented graphically in Text-fig. 1.

Hours intervening between immunization and intradermal infection	Received no sulfapyridine				Received sulfapyridine			
	Rab. No.	Incidence and duration of fever T 104° F or over	Incidence and severity of lesions	Incidence, duration and severity of bacteremia No = col./cc. D = died	Rab No	Incidence and duration of fever T 104° F or over	Incidence and severity of lesions	Incidence, duration and severity of bacteremia No = col./cc. D = died
48	8-79		++	30000 1 D	8-87		+++	30 0
	8-80		++++	0 0	8-88		+++	30 0
	8-83		++++	300 3	8-90		+++	30 0
	8-84		++++	300 3	8-91		++	0 0
	7-07		+++	300 0 0	7-01		+++	300 3 12 D
	7-08		++++	300 3 0 0	7-02		+++	300 3 12 D
	7-09		++++	15 0 0 0	7-03		+++	0 0 0 0
	7-10		++++	0 0 0 0	7-04		+++	30 0 3 0
	7-11		++++	300 3 12 11 D	7-05		+++	30 3 0 0
	7-12		+++	300 3 0 3	7-06		++	30 3 0 0
72	9-73		++	0 0	9-69		++	0 0 0
	9-74		+++	300 3	9-70		++	0 0 0
	8-55		++	0 0 0	8-20		+++	0 0 0 0
	8-58		++	30 0 0	8-28		++	0 0 0 0
	8-63		++	0 0 0	8-29		+	0 0 0 D
	8-65		+++	0 0 0	8-30		++++	99 132 3 12 3 D
	8-66		++	0 0 0	8-45		++	30 0 0
	8-71		++	30 0 0	8-48		+	0 0 0
	8-74		+++	0 0 0	8-59		+	0 30 0
	8-75		++	0 0 0	8-61		++	0 0 0
96	9-67		+	0 0 0	9-61		-	0 0 0
	9-68		+	0 0 0	9-66		+	0 0 0
	8-13		-	0 0 0	8-18		-	0 0 0 0
	8-60		-	0 0	8-22		+	0 0 0 0
	8-67		+	0 0	8-26		+++	0 0 0 0
	8-69		-	0 0	8-27		+	0 0 0 0
	8-70		-	0 0	8-39		+++	0 0 0 0
	8-72		-	0 0	8-40		-	0 0 0 0
	8-76		-	0 0	8-44		-	0 0
	8-77		++	0 0 0	8-64		-	0 0
	8-78		-	0 0				
	8-81		+++	0 0				
No. of days		1 2 3 4 5		1 2 3 4 5		1 2 3 4 5		1 2 3 4 5

TEXT-FIG. 1. Characteristics of the disease produced by intradermal infection with *Pneumococcus* Type I in rabbits previously immunized with a heat-killed suspension of organisms of the homologous type. Immunization was carried out by a single intravenous injection of a suspension of heat-killed *Pneumococcus* Type I equivalent to 10 cc. of original culture. Intradermal infection was initiated by the injection of 0.2 cc. of a 1:5000 dilution of an 8 to 10 hours rabbit blood broth culture of virulent *Pneumococcus* Type I. Solid black shading indicates the number of days during which a rabbit's temperature was elevated to 104° or above. Absence of shading indicates absence of fever. Plus signs represent the occurrence of dermal lesions graded in severity from + to represent a minimal lesion to ++++ indicating one of maximal intensity. Minus sign indicates that no lesion developed. Cross hatching represents the incidence and duration of bacteremia. The small numbers in the cross hatched areas indicate the bacterial colony counts per cubic centimeter. Cultures of the blood which proved sterile are indicated by the symbol 0. D indicates animals which died, and with but one exception is placed so as to designate the day of death. Rabbit 7-11 died on the 11th day with persisting bacteremia.

The criteria for these comparisons include the incidence and duration of fever, the occurrence and severity of the lesions, the degree and duration of bacteremia, as well as the number of fatalities and the time of their occurrence. Temperatures of 104°F. or above were considered abnormal. The degree of temperature elevation is not included in this report since in most instances high fever continued until the time of death or recovery. The severity of lesions has been designated by the symbols + to + + + +, to represent the gradations of dermal involvement varying from a small circumscribed tuberculin-like reaction to an extensive area of dependent inflammatory edema with ecchymosis and necrosis. The most severe reactions were usually observed in the unvaccinated control animals, but not invariably, since several died before the lesions appeared to be fully developed. Evidences of incipient healing of the dermal infection usually paralleled other indications of recovery. The rate of healing seemed to depend upon the extent and severity of the lesions. The outcome of the infection, however, could not be predicted from the severity of the local inflammatory process, for many of the animals infected 48 hours after vaccination had lesions of maximal intensity but ultimately survived.

In all of the control animals, and with but two exceptions among the 23 vaccinated rabbits which developed bacteremia, cultures of the blood taken 24 hours after infection yielded growth of *Pneumococcus* Type I. In most of the vaccinated rabbits, notably those with low initial colony counts, cultures of the blood became sterile coincidentally with other evidences of recovery, whereas in some of the vaccinated and in all of the unvaccinated rabbits, bacteremia persisted and progressively increased until the time of death. From seven of the eight fatalities among the 62 immunized rabbits, *Pneumococcus* Type I was isolated in cultures of the blood taken during life or at autopsy. The single exception was the rabbit mentioned above which may have died of sulfapyridine intoxication.

Non-Immunized Rabbits.—In the 26 control animals which received no sulfapyridine the infection followed a uniformly fatal course. Included in this number are those rabbits which received 1/10 or 1/100 of the inoculum employed routinely in infecting the vaccinated animals. High fever persisted usually until the time of death and the lesions were characteristically of maximal intensity. Cultures of the blood taken 24 hours after infection yielded from three to myriad colonies per cubic centimeter, and in every instance overwhelming blood stream invasion occurred prior to the invariably fatal termination within 6 days. In the four unvaccinated rabbits which received sulfapyridine the course and termination of the disease were identical.

Rabbits Infected 48 Hours after Immunization.—(Text-fig. 1.) Ten rabbits which received preliminary administration of sulfapyridine and ten which did not were infected 48 hours after immunization. As shown in Text-fig. 1 all of the untreated rabbits and all but two of those treated with the drug developed

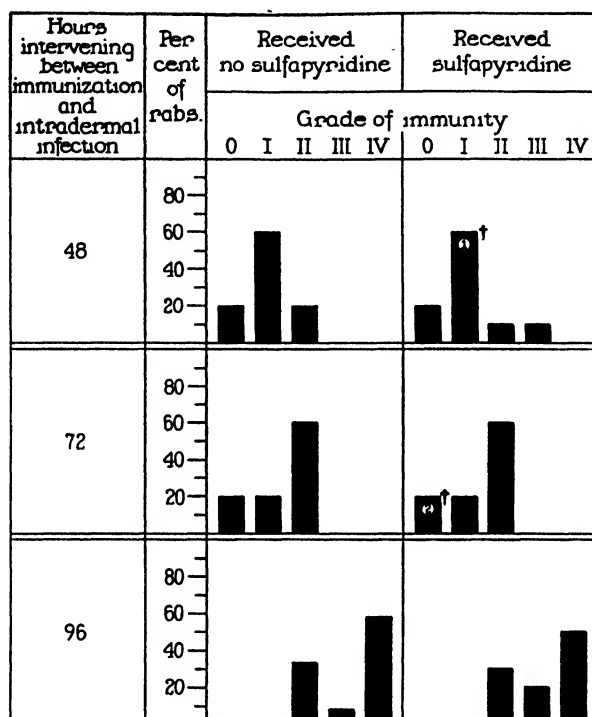
fever which lasted from 1 to 5 days, averaging about 3 days in the febrile animals of both groups. The lesions varied in severity from ++ to ++++ in both groups. On the whole the intensity of the lesions was perhaps a little less severe in the treated than in the untreated animals. However, eight of the rabbits in each group had positive blood cultures 24 hours after infection. Although the severity of illness in most of the rabbits was such that death seemed imminent over a period of several days, only two animals from each group ultimately succumbed, three within 4 days and one on the 11th day following persistent bacteremia.

Rabbits Infected 72 Hours after Immunization.—(Text-fig. 1.) In the ten treated and ten untreated rabbits which were infected 72 hours after immunization the manifestations of infection were considerably less than in the comparable groups of animals infected 24 hours earlier. Although fever occurred in every instance it was of shorter duration, averaging about $1\frac{1}{2}$ days in the untreated and $2\frac{1}{2}$ days in the treated animals. The severity of the lesions was also less intense averaging about ++ in both groups with extreme variations of + to ++++. Bacteremia occurred in four of the untreated rabbits of which two died, and in three of the treated animals of which one died. One treated rabbit which did not have bacteremia died on the 4th day of illness.

Rabbits Infected 96 Hours after Immunization.—(Text-fig. 1.) Half of the rabbits infected 96 hours after immunization, whether treated or untreated, seemed to be unaffected by the same inoculum of *Pneumococcus* Type I producing severe disease in animals infected after shorter intervals. Only four of twelve untreated and three of ten treated rabbits developed fever. The average duration was 2 days in each group. Lesions occurred in five animals of each group and were for the most part of minimal intensity with individual variations in severity to ++++. None of these animals developed bacteremia and all survived.

Active Immune Response at Different Intervals Following Immunization.—In the immunized rabbits manifestations of the disease produced by intradermal infection were progressively less severe as the interval between immunization and infection was increased. The enhancement of resistance to infection in animals which had not received sulfapyridine could be explained only on the basis of an immune response to vaccination. Likewise, in the animals which received sulfapyridine there was no evidence to contradict this assumption. In order to compare the degree of active immunity in the respective treated and untreated groups of animals, and thereby determine whether or not the response in treated animals was affected by drug administration, a modification of the classification devised by Goodner (19) has been employed. Immunized rabbits which showed all the characteristics of the disease invariably noted in the controls, including a fatal termination, were considered to have no immunity. Those which showed no manifestations of disease were re-

garded as completely immune to the infecting dose employed. Grades of immunity between these extremes have been observed as indicated in Text-fig. 2. With only two exceptions each rabbit could be readily classified in one of



TEXT-FIG. 2. Active immunity in rabbits at different intervals following immunization.

<i>Grade of immunity</i>	<i>Corresponding characteristics of disease</i>
0 = no immunity	Fever, lesion, bacteremia, and death.
I =	Fever, lesion, bacteremia, survived.
II =	Fever and lesion, no bacteremia, survived.
III =	Minimal lesion, no fever, no bacteremia, survived.
IV = complete immunity	No evidence of disease, survived.
All of the control animals had 0 immunity.	

† Only 2 animals did not fit readily into these categories: (1) moderate lesion and bacteremia, no fever, survived; (2) fever and lesion, no bacteremia, died.

the designated categories. Since all of the unvaccinated control animals showed no immunity they have not been included in the chart.

It is evident that as early as 48 hours after immunization most of the treated and untreated rabbits had some degree of resistance to intradermal infection. This is indicated chiefly by the fact that 80 per cent of the animals in both groups survived. The immunity was of a low order, however. 60 per cent

velopment of circulating antibodies as well as the early development of active resistance is not influenced by the administration of sulfapyridine.

DISCUSSION

It is evident from the experimental data that the administration of sulfapyridine has no appreciable effect upon the immune response in rabbits following a single intravenous injection of heat-killed suspension of *Pneumococcus* Type I.

Of particular significance is the fact that active immunity, as indicated by resistance to intradermal infection, was present 48 hours before any appreciable immune response could be detected by tests for circulating antibody. 96 hours after vaccination, when antibodies were first demonstrable in the sera, all of the animals tested were almost solidly immune to intradermal infection.

Of the techniques employed for the determination of circulating antibody, the mouse protection test was found to be the most sensitive, revealing the presence of antibody in approximately twice as many instances as did the test for agglutinins or precipitins.

The precipitin reaction, although an exquisitely sensitive method for the detection of minute amounts of capsular polysaccharide, is less satisfactory than the mouse protection and agglutination tests for the purpose of demonstrating small amounts of antibody. As the precipitate which forms the basis of this reaction is composed almost entirely of antibody, small amounts of antibody may fail to produce a visible reaction with varying dilutions of antigen although in the presence of adequate antibody the reaction with antigen in dilution as high as 1:5,000,000 may yield a visible precipitate. Furthermore, when used to test the sera of patients with pneumococcal pneumonia the precipitin reaction introduces another possible factor of error. If the preparation of capsular polysaccharide employed is contaminated with even a trace of the pneumococcus C polysaccharide the reaction of the latter substance with the C reactive protein of acute phase serum may be misinterpreted as an indication of the presence of type specific antibody (20, 21).

In man studies comparable to those which can be carried out in experimental animals are not feasible. Because of the many variables existing in the patient with pneumococcal pneumonia it would be impossible to conclude from the failure to demonstrate circulating type specific antibody that resistance to infection had not developed or that administration of a sulfonamide drug had affected the development of immunity.

Either failure to produce sufficient circulating antibody or the removal of free antibody by combination with circulating antigen could preclude the detection of antibody. In any event, positive results indicate only that an excess of antibody is present, whereas negative results may signify merely that such an excess cannot be demonstrated by the techniques available.

Experimental observations, however, point clearly to the fact that a considerable type specific immune response to pneumococcus develops rapidly and may be adequate to protect against infection in the absence of demonstrable circulating antibody. Although the various types of pneumococci vary in their ability to stimulate antibody formation it seems unlikely that the sulfonamide drugs exert different effects upon this particular property in different types of pneumococcus. From the present study it appears that the development of immunity in response to a specific antigenic stimulus proceeds according to the effectiveness of the stimulus irrespective of whether sulfapyridine has been administered.

SUMMARY

1. Sulfapyridine, administered to rabbits during the period of developing immunity after a single intravenous injection of heat-killed *Pneumococcus* Type I, exerted no influence upon the immune response.
2. Active immunity as indicated by increased resistance to homologous intradermal infection was present 48 hours after the immunizing injection and 2 days before circulating type specific antibodies were detectable.
3. Of the serological techniques employed for the detection of circulating antibody the mouse protective test yielded the highest percentage of positive results followed in order by tests for type specific agglutinins and precipitins, the latter being least satisfactory for the detection of small amounts of antibody.

CONCLUSION

The experimental findings lend further support to the view that, in man, effective therapy of pneumococcal infections with sulfonamide drugs is intimately associated with the development of active immunity.

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CAPACITY OF PLEUROPNEUMONIA-LIKE MICROORGANISMS TO GROW ON CHORIOALLANTOIC MEMBRANES

By HOMER F. SWIFT, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

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In recent years, the group of microorganisms resembling the etiologic agents of pleuropneumonia of cattle and of agalactia of sheep and goats has attracted considerable interest, particularly because several strains have been recovered from such commonly used laboratory animals as mice, rats, and guinea pigs and from certain non-animal sources (Klieneberger (1)). Sabin (2) demonstrated that many laboratory mice carry these microorganisms in or on their conjunctivae and nasal mucosa as apparently harmless parasites. By instilling exudates from rheumatic and other patients into the lungs of normal Swiss mice, Brown and I (3) induced pneumonia in a large proportion of animals, and from these pneumonic lungs recovered pleuropneumonia-like microorganisms belonging to Sabin Types A, B, or C. Because of Sabin's discovery of the wide-spread carrier state of mice in respect of these microorganisms, it seemed highly probable that the human exudates did not contain them; and several observers, including ourselves, have failed to grow them directly from rheumatic exudates or tissues (4-7).

During our investigations, dropped chorioallantoic membranes of incubated hens' eggs were employed in searching for suspected etiologic agents; and in one instance, pleuropneumonia-like microorganisms, recovered from the lungs of intranasally inoculated mice, apparently grew in these membranes. This strain was later found by Sabin to belong to his Type C. About the same time, Sullivan and Dienes (8) reported that they were unable to grow a strain of pleuropneumonia-like microorganisms on living chorioallantoic membranes, but they could obtain growths when the embryos were chilled to death at 4°C. This strain was identified immunologically by Sabin as his Type A. Almost simultaneously, Findlay, MacKenzie, MacCallum, and Klieneberger (9) recorded that a strain of pleuropneumonia-like microorganisms, then designated as L₇, grew well on living chorioallantoic membranes.

These discrepancies in the experience of different experimenters suggested that the ability of the pleuropneumonia-like group of microorganisms to grow on chorioallantoic membranes be tested; and this communication is the result of such an investigation.

EXPERIMENTAL

Media and Methods

Chorioallantoic Membranes.—The chorioallantoic membranes of white Leghorn eggs, incubated 10 to 15 days, usually 11 or 12, were dropped with the usual Good-pasture technique (10), following the drilling of a small hole in the shell immediately over the embryo. The shell membrane was nicked with an iridectomy knife and through this hole the inoculum in 0.1 cc. volume was introduced by means of a tuberculin syringe and a fine hypodermic needle. In case the membrane had not dropped, the needle was plunged about 1 cm. into the egg. The holes were sealed with a hot sterile vaseline-parawax mixture. Part of the eggs were incubated at 37°C. without other manipulation. The rest were chilled to kill the embryos. In the earlier experiments the eggs with dropped membranes were placed in the icebox at 5 to 8°C., for one-half to one hour, but so many embryos thrived in spite of this cooling that subsequently the eggs were chilled by placing them on paper which was resting on CO₂ ice in a heavily insulated refrigerator. When placed directly on CO₂ ice, many of the shells cracked. Because an occasional embryo survived even this refrigeration, the chilling process was finally applied for 1 hour, half before and half after inoculation, with subsequent candling to check the viability of the embryos.

Inoculum.—In a few instances, 30 per cent ascitic dextrose broth cultures were employed, but the usual inoculum was an ascitic broth suspension of ascitic agar cultures; it was prepared as follows: From heavy growths on ascitic agar in Petri dishes, several strips embracing a total area of 3 to 5 sq. cm. were cut out with a sterile platinum spud and transferred to a pointed centrifuge tube; the agar was finely minced with the spud, then mixed thoroughly with 1 to 1.5 cc. of 30 per cent ascitic broth. After standing a few minutes, the tube was centrifuged at low speed for 4 minutes. The supernatant fluid was decanted onto the higher side of a slightly sloping Esmarch dish; in this way many small bits of suspended agar were caught on the glass, and the fluid which collected at the bottom of the slope could be easily aspirated through a fine needle into a Luer syringe. Even with these precautions, many minute particles of agar were carried along with the inoculum. The micro-organism content of this inoculum was roughly determined by inoculating a 30 per cent ascitic agar plate with a drop of the fluid from the syringe, and determining the amount of growth after 4 days incubation.

Media for Growing Pleuropneumonia-Like Microorganisms.—In our hands, 30 per cent ascitic agar and 30 per cent ascitic broth have proven uniformly the most satisfactory media for growing all strains of pleuropneumonia-like microorganisms that have been available. The broth was an infusion of beef heart made in a proportion of 450 gm. of lean meat per liter of water, neopeptone 1 per cent and NaCl 0.5 per cent. The final pH was 7.8. To this broth was added 30 per cent of suitable ascitic fluid, or other sera, after it was filtered through a Seitz E K pad under 20 lbs. pressure. In some instances, the media was further enriched with 0.5 to 1 per cent dextrose in sterile solution. After incubation to test sterility, the ascitic broth was stored in the refrigerator.

The 2 per cent nutrient agar was prepared from beef heart infusion with 1 per cent neopeptone and 0.5 per cent NaCl and adjusted to a final pH of 7.8. To flasks of this

agar melted and cooled to 45°C. was added 30 per cent of Seitz filtered warm ascitic fluid. This mixture was poured into clear glass Petri dishes having *fine filler paper* in the tops to absorb condensed water. When the media was well solidified, the Petri dishes were sealed with parafilm in strips about 2 cm. wide, pressed well on the glass with a photograph squeegee roller. After 24 to 36 hours incubation, the plates were examined microscopically and stored on a laboratory table (not in the refrigerator). Before being inoculated, the medium was again carefully examined either with a hand lens or low power microscope and those plates showing bacteria or spores of moulds were discarded. It was important to use plates not over 7 to 10 days old, because of the tendency of older ones to develop numerous pseudocolonies of spherocrystals (11).

These plates were inoculated in a bacteriological hood or in a tissue culture room having filtered air, and resealed with fresh parafilm. This seal was an important feature of the technique, for when properly made it insured a moist atmosphere in the Petri dish during both the preliminary incubation and storage of the media and following the inoculation.

The material to be tested for content of pleuropneumonia-like microorganisms consisted of a pulp of the chorioallantoic membrane or embryo, minced finely with a scissors. This was streaked over the surface of the media with the convex surface of a slightly curved spud, which was more satisfactory than the usual platinum loop. In making subcultures, a suitable area of the agar was cut out, lifted to a fresh plate with a spud, and the colony-bearing surface of the "cutout" was placed in contact with the upper surface of the fresh medium; then the "cutout" was carefully rubbed over the surface of the ascitic agar with the spud. The tendency for pleuropneumonia-like microorganisms to grow down into the agar makes necessary this mode of transferring, described by Klieneberger (12), for it is difficult and often impossible to transfer this type of colony by the usual bacteriological technique. In subculturing these cultures into liquid media a "cutout" of the agar, bearing colonies, was transferred to the ascitic broth with the spud. When subcultures were made from fluid to solid media, a drop of the ascitic broth culture was placed on the surface of the ascitic agar and the cover of the Petri dish was immediately replaced; then the drop of fluid was spread over the surface of the medium by properly tipping the dish, with care not to allow the fluid to touch the sides. In all of these maneuvers, it was important to reduce the period during which the plates were opened to the shortest possible interval, for air borne bacteria and spores of moulds were frequent sources of contamination; and once contaminated with moulds, it was very difficult to purify the cultures of pleuropneumonia-like microorganisms without resorting to filtration.

The advantage of the above described technique is that the growth and development of the colonies can be followed with the low power microscope (objective 10×, ocular 10×) without opening the Petri dish and running the great risk of contamination, for the characteristic colonies usually do not become visible before the 3rd day, and sometimes not until the 5th to 7th, particularly when grown from tissues. For the most satisfactory microscopic examination, a fairly strong artificial light is desirable; the substage condenser is elevated as high as possible and the beam of light made very fine by almost closing the iris diaphragm. The Petri dish is placed with the top down on the microscope stage and a fine beam of light is focused sharply on the filter paper in the Petri dish. This gives about the proper illumination of the colonies

which are viewed through both the bottom of the dish and the agar; hence the importance of having these light-transmitting media crystal clear. By slightly tipping the substage mirror from side to side, or by moving the lamp, the beam of light can be made to strike the colonies obliquely and thus their texture and the character of the centers can be better brought out. These colonial appearances are rather characteristic for the different types, as emphasized by Klieneberger (12). Small areas on the medium can be identified by marking the overlying surface of the Petri dish with a glass-marking pencil; and isolated individual colonies can be marked by fastening a bit of perforated paper directly over the colony with Scotch tape; if the marker is properly placed, the colony can be repeatedly studied through the perforation; and numbered paper can be used to identify a particular area or colony for transfer.

Obviously for this technique the media must be crystal clear; hence blood agar or Klieneberger's special media (13) for growing pleuropneumonia-like microorganisms is not usable, even though it efficiently nourishes these microorganisms. Neither is clear media slanted in tubes so satisfactory, because of the difficulty in microscopic examination, which is necessary to detect such minute colonies. In our experience, the most important component of this media is proper ascitic fluid in 30 per cent concentration. Not all ascitic fluids are satisfactory; hence it is advisable to test each new fluid for its capacity to sustain all available pleuropneumonia-like microorganisms before adopting it for routine use. With these precautions, we have grown all of the strains of pleuropneumonia-like microorganisms that have come to our hands even though several of them had been grown by Klieneberger for many months on media enriched with horse or beef serum. For many strains, horse-, beef-, or rabbit-serum agar are satisfactory enriching substances, but because ascitic fluid in 30 per cent concentration has been found requisite to grow others, it seemed desirable to adopt this as a routine media for recovering pleuropneumonia-like microorganisms from tissues. With it we have easily recovered strains directly from pneumonic mouse lungs, from apparently normal mouse conjunctivae, and from the chorio-allantoic membranes used in the present work. Occasionally, only 3 to 10 colonies could be found on an entire plate, numbers that could easily have been overlooked on any other media. Moreover, it has been possible to obtain subcultures from "cutouts" containing one or two colonies. Finally, the relative number of colonies on a plate inoculated with minced tissue has given a rough index of the number of microorganisms in that tissue compared with others.

Microscopic study of pleuropneumonia-like microorganisms is most easily made in Giemsa-stained films and in dark field preparations of fluid cultures such as the 30 per cent ascitic broth, but it sometimes takes much longer to establish cultures of these microorganisms in fluid than on solid media, and then only after many "blind transfers." Moreover, some strains die much more rapidly in fluid cultures than on solid media; and contaminants can be more readily separated when they appear on agar, although it is fairly easy to purify fluid cultures by suitable filtration.

Strains of Pleuropneumonia-Like Microorganisms Tested.—Eight different strains belonging to four different immunological types were tested for their ability to grow on chorioallantoic membranes.

L₄ Strains.—*L₄* obtained from Miss Klieneberger was originally isolated from a rat's enlarged lymph node. It had been carried through many subcultures, on various media.

L_{7a} and L_{7b}, obtained from Dr. Angevine, were derived from the L₇ strain isolated from arthritic joints of rats by Findlay, MacKenzie, MacCallum, and Klieneberger (9). Later Klieneberger (1), determined that this strain was immunologically similar to L₄. Dr. Angevine had repeatedly subcultured the two variants in different media, the *a* variant on ascitic agar and the *b* variant in ascitic broth, and had found different colonial forms in these variants when they were subcultured on ascitic agar.

L₅ Strains.—L₅, obtained from Klieneberger, isolated by Findlay, Klieneberger, MacCallum, and MacKenzie (14), from the brain of a mouse with rolling disease.

Sabin Type A. Strain A78, isolated from mice by Sabin (15, 16). Young cultures, or filtrates of young cultures, induced rolling disease in mice and cultures inoculated intravenously induced polyarthritis.

Strain CA 95, isolated by the author from pneumonic lung of a mouse which had received intranasal instillations of a rheumatic exudate culture agglutinated in high titre in Type A serum. Klieneberger (1) has shown that Sabin Type A and her L₅ are similar immunologically.

Sabin Type B.—Strain B43, obtained from Sabin as a representative strain of this type, was isolated originally from Rockefeller Institute stock of albino mice.

Pleuropneumonia contagiosa bovis.—Strain P₁ was originally from London type collection, but had been maintained many years on artificial media. Recently kept in desiccated state after freezing.

All of the strains when grown on 30 per cent ascitic agar showed on microscopic examination colony forms of isolated colonies similar to those described and pictured by Klieneberger (12).

RESULTS

The results of testing the four different types, embracing eight different strains or variants, are given in detail in Table I. There were eighteen satisfactory experiments; but in many others in which bacterial contaminants or moulds rendered estimation of the comparative growth of pleuropneumonia-like microorganisms on the ascitic agar plates less accurate, the same tendencies were noted.

Under the experimental conditions, three categories of embryonic growth conditions are classifiable: A, those with embryos alive both at the times of inoculation and final examination; AD, those alive when inoculated but dead when examined; and D, those dead when inoculated. Group A included a few where the chilling did not kill the embryo, which then lived and developed like their unchilled mates. In group AD, most of the embryos died shortly after the membranes were inoculated; hence from the standpoint of furnishing living or dead tissue for growing pleuropneumonia-like microorganisms, they must be considered almost comparable with group D.

It is unlikely that the pleuropneumonia-like microorganisms killed these dead embryos, for if these infectious agents were in themselves lethal, it is improbable that over three-fourths of the total inoculated living embryos would have survived. The fact that there is usually some mortality among embryos

TABLE I

Type of pleuropneumonia-like microorganism	Strain	Exp. No.	Media which inoculum for embryos was grown	Categories of embryos					
				A		AD		D	
				Alive when inoculated	Alive when examined	Alive when inoculated	Dead when examined	Dead when inoculated	Dead when examined
L ₄	L ₄	14	AA†	-	++	++	++	++	++
	"	15	AA	-	++	+++	+++	+++	+++
	"	18	AA	++	+++	+++	+++	+++	+++
	"	20	AA	++	+++	+++	+++	+++	+++
	L _{7a}	31-2	AA	+++	+++	+	+++	+++	+++
	L _{7b}	33-4	AA	+++	+++	+++	+++*	+++*	+++*
	L ₄	12-13	AB	-	+++	±	+++	+++	+++
L ₄	"	19	AA	-	+++	+++	+++	+++	+++
	"	21	AA	(±)	+++	+++	+++	+++	+++
	A78	16-17	AA	-	+++	+++	+++	+++	+++
	CA 95	37	AA	(±)	+++	+++	+++	+++	+++
	"	38	AA	-	+++	+++	+++	+++	+++
	"	22	AA	±	+++	+++	+++	+++	+++
<i>Pleuropneumonia contagiosa bovum</i>	B43	23	BB	+++	+++	+	+++	+++	+++
	"	24	AA	±	+++	+++	+++	+++	+++
	"	25	AA	+++	+++	+++	+++	+++	+++
	"	26	AB	+	+++	+++	+++	+++	+++
	"	27	AA	+++	+++	+++	+++	+++	+++

† AA = ascitic agar.

AB = ascitic broth.

BB = beef serum broth.

* Growth formed a solid band.

(±) to +++ indicates relative amount of growth on ascitic agar plates inoculated with minced chorioallantoic membranes.

-- indicates no growth on ascitic agar plates inoculated with minced chorioallantoic membranes.

in eggs submitted to the membrane dropping technique probably accounts in large part for the deaths in group AD.

In five eggs belonging to group A, the inoculum was injected into the eggs because it was impossible to drop the membranes satisfactorily; as the recovery of pleuropneumonia-like microorganisms in all such instances was comparable to that from dropped membranes the results are combined in the table. From blood-free allantoic or amniotic fluid of several inoculated eggs, it was possible to grow pleuropneumonia-like microorganisms in concentrations almost as heavy as from the corresponding membrane. It therefore seems probable that all tissues bathed by these infected fluids contained these microorganisms.

A casual glance at Table I reveals that there was no general rule concerning the ability of pleuropneumonia-like microorganisms to grow on living chorio-allantoic membranes. Three of the four types tested grew well under such vital conditions, but the fourth, Klieneberger's L₆, usually did not survive. Nevertheless, from four out of fourteen living membranes inoculated, respectively, with three different representatives of Type L₆, it was possible to recover a very few colonies on ascitic agar plates. These few colonies would doubtless have escaped detection with any other technique than that which made possible a thorough microscopic examination of the entire agar surface. Whether this slight infection of the membranes would have been detectable by a series of "blind passages" through fluid cultures, it is impossible to state. Certainly it would have been difficult to make such comparative studies with fluid media. One may properly question whether in these exceptions with Type L₆, the survival of the few microorganisms in the membranes might not have been due to their presence in bits of agar carried along in the fluid with which the eggs were inoculated. No data are available to throw light on this question; although with other types good growths occurred on living membranes inoculated with ascitic- or beef serum-broth cultures free of agar. It is interesting that strains L₆, isolated by Klieneberger, A78, isolated by Sabin, and CA 95, isolated in our laboratory, all behaved similarly in their growth on chorioallantoic membranes, and also much like the Sabin Type A strain, studied by Sullivan and Dienes (8). Their similarity in this respect is confirmatory of the type relationship of these strains, established immunologically by Klieneberger and Sabin, respectively.

From living membranes inoculated with strains belonging to Klieneberger Type L₄, Sabin Type B, and *Pleuropneumonia contagiosa bovis*, good growths were obtained; therefore, in sharp contrast with Type L₆, it is obvious that the majority of types tested were capable of growing on such membranes. This capacity, however, varied: it was less marked in the strains belonging to Types L₄ and B than in *Pleuropneumonia contagiosa bovis*, which incidentally induced the most marked macroscopic and microscopic evidence of growth in these viable membranes.

Even though these three types grew well in living membranes, they multiplied in all experiments much better in dead ones. This is best illustrated by reassembling the data in the form shown in Table II, where it is evident that the best membranes for growing all strains were those which were dead when inoculated, *i.e.* category D; next in order were those that died shortly after being inoculated (AD). Indeed, from several of the killed membranes inoculated with strains belonging to strains L₇ (Type L₄) and *Pleuropneumonia contagiosa bovis*, there was obtained on the ascitic agar plates a growth so heavy that it formed a grayish band on the surface of the medium; and the typical colonies were recognizable only on thinly inoculated areas or on subcultures. From these results, it seems evident that if the chorioallantoic

TABLE II

Type of pleuropneumonia-like microorganism	Category	Total	Degree of growth on ascitic agar plates						
			-	(±)	±	+	++	+++	++++
L ₄	A	12	2	8	2
	AD	7	.	.	1	1	1		4
	D	12	12
L ₅	A	14	10	3	1
	AD	1	.		1	.		.	.
	D	10	.					.	10
Sabin B	A	5			1	1	2	1	.
	D	1	.						1
<i>Pleuropneumonia contagiosa bovis</i>	A	11		..	1	1	2	3	4
	AD	5	..			1	1	1	2
	D	7	..		1	.		2	4

membrane technique is to be employed in searching for the presence of pleuropneumonia-like microorganisms in tissues or exudates, a better chance of recovering them would be afforded by killing the embryos aseptically before placing the suspected material on the membranes.

Incidentally, the effect of growing variants of pleuropneumonia-like microorganisms on chorioallantoic membranes was studied. This was suggested by some observations of Dr. Angevine (17) from whom strains L_{7a} and L_{7b} were obtained. The L₇ strain had been sent to him by Miss Klieneberger. In culturing the strain on ascitic agar and in ascitic broth respectively and occasionally comparing the morphology of the colonies in subcultures on ascitic agar, Angevine noted that variants developed in the liquid media, which tended to retain their variant morphology when repeatedly subcultured on solid media. When, on the other hand, their pathogenicity was tested in animals, the colo-

nies obtained from the lesions were always of the original type, here designated as *a*; the variants which developed in ascitic broth are designated as *b*. In six subcultures on ascitic agar in our laboratory, the two strains retained their respective colonial appearance. The *a* colonies were round; only moderately granular; the margins were distinct; and the central portions were relatively small and fairly translucent. The *b* colonies, on the other hand, were larger and appeared more granular; the margins were somewhat indistinct; the centers were two to three times the size of the centers of the *a* colonies and were distinctly more granular.

Both types of variants grew well on either living or killed chorioallantoic membranes; but infinitely better growth was obtained from the dead ones (experiments 31-2, 33-4). The ascitic agar cultures from all of the membranes, however, presented the morphology of the *a* colonies. Thus the passage of variants through chorioallantoic membranes had the same influence in causing them to revert to the original form as had passage through such mammals as rats and mice. The danger of contamination with other strains of pleuropneumonia-like microorganisms in passing through eggs is, of course, much less than in passage through rats and mice, which are now known to be frequent carriers of this class of microorganisms.

Lesions Induced in Chorioallantoic Membranes

No constant lesions were observed in the living membranes inoculated with the several strains of pleuropneumonia-like microorganisms. Many of the membranes showed gray central areas of thickening which were probably induced in large part by the technical procedures. Only rarely were there small diffusely-scattered white spots which resembled somewhat the lesions described by Burnet (18) as pox. About one-fourth of the membranes were thin and transparent; half showed varying degrees of edema, in some very marked; but no relationship could be determined between the amount of edema and the intensity of infection as determined by the content of microorganisms. All of the living membranes infected with strain L₇ were from 1 to 3 mm. thick, and had a gelatinous consistency. Half of the membranes infected with *Pleuropneumonia contagiosa bovis* had a similar thick gelatinous or leathery appearance.

Only a few of the markedly thickened membranes were examined microscopically, and no attempt was made to study the histological evolution of lesions. It is doubtful whether the abnormal microscopic picture could all be attributed entirely to the action of the microorganisms, for the inoculum often contained numerous minute particles of agar which doubtless induced foreign body reactions in the membranes. The edematous membranes showed some increase in mesodermic cells which were widely separated by clear spaces; the gelatinous membranes often revealed a distinctly increased number of fibro-

blast-like cells; and in those membranes infected with strain L₇ there were numerous scattered accumulations of large round cells with a heavily staining nuclei. Those infected with *Pleuropneumonia contagiosa bovis*, on the other hand, although just as abnormal in the gross, showed much less round cell infiltration in the mesoderm. Because of the great variability in both macro- and microscopic appearance, it is doubtful whether any of the alterations had pathognomonic significance. To determine the chorioallantoic tissue reactions to infection with various strains of pleuropneumonia-like microorganisms, a much more systematic investigation should be pursued with inocula free of particulate foreign bodies.

DISCUSSION

As suggested by the literature, considerable variability was found to exist among different pathogenic strains of pleuropneumonia-like microorganisms in their ability to grow on living chorioallantoic membranes. While the present experiments employed only a part of the known strains of this class of microorganisms, it is evident that no universal rule can be laid down concerning the ability of this class of microorganisms to survive under the peculiar avian embryonic conditions that are favorable for so many bacteria and viruses. From our experience with the three strains of Klieneberger L₄ type (Sabin Type A) examined by us and the one strain tested by Sullivan and Dienes, it appears that immunologically related strains probably have similar capacities for growing on chicken chorioallantoic membranes. On the other hand, within Type 4, strain L₇ induced much more marked lesions in the membranes than did strain L₄, even though both grew with comparatively the same facility in these tissues. No relationship, however, could be detected between a given strain's capacity for inducing lesions in mammals and its ability to multiply in chick chorioallantoic membranes. Indeed, the capacity for these mammalian pathogenic microorganisms to induce marked or characteristic lesions in either the chick embryo or membranes seemed to be minimal. It is doubtful whether any of the strains tested had a lethal effect on these chick embryos, for whether they grew luxuriantly or poorly on the membranes, by far the majority of the embryos survived, and showed no characteristic macroscopic lesion when examined.

It is of interest that passage through chorioallantoic membranes caused a morphological variant L_{7b} to reassume its original colonial morphology in the same manner as passage through its normal host, the rat. This observation suggests that the egg embryo passage technique might be more reliable in studying the ability of strains of pleuropneumonia-like microorganisms to assume their characteristic colonial morphology than would passage through rodents, for it is now well recognized that rodents may harbor many different

types of these microorganisms in an apparently latent form; and this carrier state makes it possible to contaminate a given strain of pleuropneumonia-like microorganisms with strains of another variety if it is passed through such animals.

The rule that sterile killed chorioallantoic membranes support the growth of pleuropneumonia-like microorganisms better than living ones is worthy of note; in this respect they behaved more like bacteria than like viruses, which practically always require living cells for their growth. Possibly sterile dead chorioallantoic membranes would furnish the most suitable medium for pleuropneumonia-like microorganisms of fastidious growth demands. Certainly killed membranes should always be used along with living ones, if this technique is employed in searching for pleuropneumonia-like microorganisms in tissues suspected of harboring them.

SUMMARY

1. Among eight strains or variants, included in four different immunological types of pleuropneumonia-like microorganisms, all grew on chorioallantoic membranes; those belonging to Klieneberger's Type L₈ (Sabin Type A) grew very poorly; and those included in three other types grew with varying degrees of vigor.

2. In all instances, strains of pleuropneumonia-like microorganisms tested grew better on dead sterile chorioallantoic membranes than on living membranes.

3. None of the strains tested was in itself lethal for chick embryos.

4. No constant macroscopic lesion developed as a result of inoculating chorioallantoic membranes with pleuropneumonia-like microorganisms.

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FURTHER STUDIES ON THE SEROLOGICAL REACTIONS OF THE SOLUBLE ANTIGENS OF INFECTIOUS MYXOMATOSIS

BY J. DE CASTRO TEIXEIRA AND J. E. SMADEL

(From the Hospital of The Rockefeller Institute for Medical Research)

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The specific soluble substances associated with certain viruses are found, in general, in tissues of each susceptible host that has been studied. This has been clearly demonstrated in vaccinia (Craigie and Wishart, 1935 and Smadel and Wall, 1937), and in lymphocytic choriomeningitis (Smadel, Baird and Wall, 1939); furthermore, the statement appears to be true regarding psittacosis (Bedson, 1936 and Lazarus and Meyer, 1939), and influenza (Hoyle and Fairbrother, 1937, and Lennette and Horsfall, 1940, 1941), although the experimental data bearing on this precise point are somewhat indirect. Reports on the precipitinogen of yellow fever, which is usually grouped with the soluble antigens, do not deal with the distribution of the substance in tissues of various susceptible species (Hughes, 1933).

Blood and infected tissue of rabbits suffering from infectious myxomatosis have been shown to contain two specific soluble antigens separable from the virus (Smadel, Ward and Rivers, 1940) which have been designated A and B. The pathogenicity of this virus is limited to rabbits, to some species of hares (Moses, 1911), and to embryonated eggs (Lusk, 1937); therefore, it seemed of interest to determine whether infected chick embryos contained one or both of the soluble antigens. The results of experiments bearing on this point will be presented at this time. In addition, observations will be recorded concerning the complement-fixing power of preparations of active and degraded soluble antigens of myxoma and of suspensions of active and inactive elementary bodies of this disease. Finally, experiments will be discussed

which can be interpreted as indicating that degraded soluble antigens may constitute one of the heat-stable agglutinogens of the virus particle.

MATERIALS AND METHODS

Suspensions of washed elementary bodies of myxoma and of virus-free solutions of antigens were obtained from infected rabbits and fertile hens' eggs. The techniques employed for the preparation and testing of materials derived from rabbits has been described in detail elsewhere (Rivers and Ward, 1937, and Smadel, Ward and Rivers, 1940). Chorioallantoic membranes of 11- to 13-day old embryonated eggs were inoculated initially with a bacteriologically sterile suspension of infected subcutaneous tissue from a rabbit with myxomatosis; subsequently, membranes were inoculated with 5 drops of a 1:200 dilution of a suspension of infected egg membrane and harvested after incubation at 37°C. for 72 hours. Experiments were carried out with material derived from the 2nd to 39th subcultures of the virus. Methods previously used to obtain washed elementary bodies and soluble substances from chorioallantoic membranes infected with the virus of vaccinia (Smadel and Wall, 1937) were applied without modification to the present problem. In a few instances, *i.e.*, those in which washed viruses were used in complement-fixation studies, tryptic digestion of the virus-laden first sediment obtained by angle centrifugation was omitted. Moreover, in several experiments ultracentrifugation (Bauer and Pickels, 1936) at 30,000 R.P.M. for 20 minutes was substituted for filtration through a Seitz pad, a procedure which was used to remove residual virus from the solutions containing the A and B antigens. The infectivity of preparations derived from eggs was determined by means of intracutaneous inoculations in rabbits of .25 ml. of serial tenfold dilutions of the test material.

The details of the methods employed in the agglutination and precipitation tests have been recorded (Rivers and Ward, 1937). Complement-fixation tests were carried out by the technique used in studies on lymphocytic choriomeningitis (Smadel, Baird and Wall, 1939), *i.e.*, test mixtures of antigen and antibody containing

two units of complement were stored overnight at 0°C. and then the hemolytic system was added.

Antisera used throughout the present work were of two types; (1) anti-soluble-substance sera obtained from rabbits immunized with virus-free solutions of antigens of myxoma, and (2) immune serum from a rabbit convalescent from infection with the virus. The antisera are here designated by the same numbers employed earlier (Smadel, Ward and Rivers 1940).

EXPERIMENTAL

Observations on suspensions of elementary bodies and solutions of antigen from infected chorioallantoic membranes

It may be stated at once that in our hands the preparation of washed virus and of soluble antigen from chorioallantoic membranes infected with the virus of myxomatosis was less successful than it was in earlier experiments with eggs infected with the virus of vaccinia (Smadel and Wall, 1937). This was true even though the intensity of the chorioallantoic lesions and the infectivity of crude suspensions of diseased membranes were comparable following inoculation with the two viruses. Washed elementary bodies of myxoma, whether derived from rabbits or eggs, display a greater tendency to undergo auto-agglutination than do elementary bodies of vaccinia. We have assumed that aggregation of elementary bodies with resultant loss of virus during the process of washing and differential centrifugation was mainly responsible for the reduction in infective titer. The results of several experiments summarized in Table I indicate that the infective titers of crude membrane suspensions were 10^{-7} to 10^{-8} , whereas those of suspensions of thrice washed virus obtained from these crude preparations were 10^{-5} to 10^{-6} .

The comparatively low infectivity of suspensions of washed elementary bodies of myxoma prepared from membranes would indicate that too few virus particles were present, according to Merrill's hypothesis (1936), to obtain a visible serological agglutination when tested by the method we have employed. In any case, negative results were obtained in agglutination tests with these suspensions. (See table 1.)

Specific soluble precipitable substance of myxoma was demonstrable in extracts of infected membranes that had been freed of virus by Seitz filtration or by ultracentrifugation at 30,000 R.P.M. for 20 minutes. Precipitin tests were performed by mixing graded dilutions of antiserum with constant amounts of undiluted solutions of antigen; this was done because the amount of soluble antigen in membrane filtrates was too small to be detected by this technique if the antigen was diluted appreciably. The applicability of the precipitin reaction was limited in filtrates of

TABLE 1

Virus and soluble antigen content of chorioallantoic membrane and embryo tissue infected with the virus of myxoma

EXPERIMENT	MATERIAL	EGG PASSAGE	CRUDE SUSPENSION		SUSPENSION OF ELEMENTARY BODIES (WASHED 3 TIMES)			SOLUTION OF VIRUS-FREE ANTIGEN	
			Volume	Infectivity	Volume	Infectivity	Agglutination titer	Precipitin titer*	Complement-fixation titer†
			ml.		ml.				
1	12 membranes	20th	10	10^{-7}	5	10^{-5}	0	1:8	1:16
2	20 membranes, 5 embryos	37th	28	10^{-8}	7	10^{-6}	0	1:4	1:16
		37th	30	10^{-4}				0	1:2
3	18 membranes, 4 embryos	39th	24	10^{-8}	7	10^{-5}	0	1:8	1:16
		39th	30	10^{-4}				Not done	1:2

* Dilution of serum.

† Dilution of antigen.

this type, since at least half of the preparations contained material which was precipitated non-specifically in control tests with normal rabbit serum. Consequently, a more satisfactory method was sought, and it was found in the complement-fixation technique. Filtrates of infected membranes regularly fixed complement when dilutions as great as 1:16 were mixed with appropriately diluted antiserum. The filtrates were practically never anticomplementary even in the undiluted state nor did they react with normal rabbit serum. A summary of the results obtained in several instances by precipitation and complement-

fixation techniques is included in table 1. It is also evident from the data included in the table that in the present experiments infected embryos contained much less virus and soluble antigen than did the membranes. Four antisera were used in this portion of the work, namely, anti-soluble substance sera numbers 68, 75 and 9212, and convalescent serum number 1; all were shown to contain antibodies against both A and B soluble antigens in experiments which were being conducted at this same time and which have been reported recently (Smadel, Ward and Rivers, 1940). The above observations indicate that at least one of the soluble antigens of myxoma is present in infected membranes but they failed to prove that both antigens are present.

The specific precipitable substance in filtrates of infected membranes was readily removed by a single absorption with optimal amounts of antisera. In these experiments undiluted filtrate was mixed with diluted anti-soluble-substance serum and incubated at 37°C. for several hours; finally the precipitate which had formed was removed by centrifugation. The resultant mixture was tested for residual antigen by the complement-fixation technique.

Absorption of antibody by antigen in filtrates was also demonstrated but it was necessary to use large amounts of filtrate in order to absorb completely all of the anti-soluble substance antibodies of both the A and B types. The presence or absence of residual antibodies after absorption was determined by testing the capacity of the mixtures to precipitate in the presence of varying dilutions of solutions of partially purified A and B antigens of myxoma which had been prepared from extracts of infected skin of rabbits. Antisera numbers 9212 and 75 were absorbed with filtrates of suspensions of egg membranes from passages 6, 7, 14, and 18; the results of an illustrative absorption experiment are presented in table 2. None of the absorbed mixtures contained residual antigen as determined by complement-fixation titrations. Antiserum 9212 contained about equal amounts of A and B antibody, *i.e.*, a 1:16 dilution of serum gave a precipitate in the presence of optimal amounts of a solution of each antigen. The results presented in table 2 would suggest

either that antigen B is present in smaller amounts than is antigen A in filtrates of infected membranes, or that B antibody is more difficult to absorb than A. Some evidence for the latter belief may be obtained from data presented in an earlier communication (Rivers, Ward and Smadel, 1939). The important conclusion to be drawn from these absorption experiments is that filtrates of infected egg membranes contain both A and B antigens of myxomatosis.

TABLE 2

Absorption of anti-soluble substance serum with filtrate of infected membrane

ANTISERUM	ANTI-GEN*	DILUTION OF ANTIGEN					
		1:2	1:4	1:8	1:16	1:32	1:64
9212 (dilution 1:8)	A	++	++++	++++	+++	+	±
	B	+++	++++	++++	+++	++	—
9212 absorbed with 4 volumes of filtrate†	A	—	—	—	—	—	—
	B	++	++	++	++	—	—
9212 absorbed with 7 volumes of filtrate†	A	—	—	—	—	—	—
	B	—	—	—	—	—	—

* Antigens A and B were prepared from virus-free extracts of skin of rabbits infected with myxomatosis. Solution A represented that portion of the globulin fraction which was insoluble at pH 4.5 in a one-third saturated solution of ammonium sulfate. Solution B contained the remainder of the globulin.

† Dilution of original serum, 1:8.

Complement fixation with heated solutions of antigen

The A and B soluble antigens of myxoma have been considered to be heat labile, since heating at 56°C. for $\frac{1}{2}$ hour destroys their specific precipitability. Nevertheless, solutions of heated antigens are still capable of reacting with their antibodies as has been demonstrated by means of the inhibition technique (Smadel, Ward and Rivers, 1940).

Active filtrates of infected membranes, like preparations of antigen from rabbits, were rendered non-precipitable by heating at 56°C. In contrast, the titer of heated filtrates when determined by the complement-fixation technique was essentially the same after heating as it was before. Dermal filtrate prepared

from myxomatous rabbits was likewise found to have approximately the same complement-fixing titer whether fresh or heated at 56°C. The results of several typical experiments are summarized in table 3. Subsequently, complement-fixation tests on partially purified solutions of A and B antigens obtained from infected skin indicated that these also behaved in a similar manner. These observations suggest that although the changes induced in the antigens of myxoma by a temperature of 56°C. are

TABLE 3

Results of complement-fixation tests with fresh and heated filtrates of infected membrane and rabbit skin

FILTRATE	NUMBER	TREATMENT	DILUTION OF ANTIGEN				
			1:2	1:4	1:8	1:16	1:32
Chorioallantoic membrane	10	Fresh	++++	++++	++++	++	
		Heated 56°C. ½ hour	*	++++	++++	++++	
	21	Fresh	++++	++++	++++	++++	
		Heated 56°C. ½ hour	*	++++	++++	++++	
Rabbit skin	841	Fresh		++++	++++	++++	+++
		Heated 56°C. ½ hour		*	++++	++++	++++
	974	Fresh		++++	++++	++++	++++
		Heated 56°C. ½ hour		*	++++	++++	++++

Anti-soluble substance serum 75 was used in a dilution of 1:32 throughout the experiment.

One to four plus represent degrees of fixation.

* These dilutions were slightly anticomplementary.

sufficient to change their precipitability they are nevertheless comparatively mild.

Complement fixation with unheated and heated suspensions of washed elementary bodies obtained from infected membranes and skin

Suspensions of infective and heat-inactivated elementary bodies of myxoma were next tested for their capacity to fix complement with anti-soluble-substance serum and myxoma immune serum.

The results of experiments summarized in table 4 indicate that suspensions of elementary bodies of myxoma prepared from embryonated eggs or from rabbits and tested with either of the two types of antisera fixed complement to approximately the same degree whether active or heated.

TABLE 4

Results of complement-fixation tests with fresh and heated suspensions of elementary bodies

E.B. PREPARATION	TREATMENT	RABBIT SERUM*	DILUTION OF E.B. SUSPENSION						
			1:4	1:8	1:16	1:32	1:64	1:128	1:256
Chorioallantoic membrane Lot 21	Fresh	75	++++	++++	++	++	+	-	
		1	++++	++++	++++	++	++	-	
	Heated 56°C. ½ hour	75	++++	++++	+++	++	-	-	
		1	++++	++++	+++	++	-	-	
		Normal	-	-	-	-	-	-	
Rabbit skin Lot 974	Fresh	75	++++	++++	++++	+++	++	++	-
		1	++++	++++	++++	++++	++	+	-
	Heated 56°C. ½ hour	75	++++	++++	++++	++	+	+	-
		1	++++	++++	++++	+++	+	+	-
		Normal	-	-	-	-	-	-	-

* Antiserum 75 obtained by immunizing rabbit with non-infectious extract of myxomatous skin. Antiserum 1 obtained from rabbit convalescent from myxomatosis. All sera were used in a dilution of 1:32 throughout experiment.

One to four plus represent degrees of fixation.

Observations on agglutination of collodion particles coated with heat-degraded soluble antigen A by anti-soluble-substance sera

Suspensions of elementary bodies of myxoma prepared from skins of rabbits are agglutinated equally well by fibro-myxoma and myxoma immune sera whether the virus is employed in the infective state or has been previously inactivated by heating at 56°C. for 1 hour (Rivers, Ward and Smadel, 1940). Furthermore, active elementary bodies which are agglutinated by anti-soluble-substance serum are still agglutinated by such antiserum after heating, but the titer is considerably reduced. The observations on the complement-fixing power of heated soluble

antigens and virus suggested that non-precipitable forms of the soluble antigens might still be capable of contributing to the flocculation of heated elementary bodies by antisera. This hypothesis was tested in the following manner.

A solution of partially purified antigen A was obtained from a Seitz filtrate of a suspension containing the ground infected skins of 2 rabbits. The preparation contained that portion of the globulin fraction which was insoluble in a solution containing 30 per cent ammonium sulfate and .01 M acetate buffer, pH 4.5. Part of the solution was heated at 60°C. for 1 hour and subse-

TABLE 5

Flocculation of collodion particles treated with unheated and heated A antigen

ANTIGEN	TREATMENT	RABBIT SERUM	DILUTION OF SERUM					
			1:2	1:4	1:8	1:16	1:32	1:64
A antigen unheated*	No collodion	75	++++	++++	+++	++	-	-
	Collodion	75	++++	++++	+++	++	±	-
A antigen heated 60°C. 1 hour*	No collodion	75	-	-	-	-	-	-
	Collodion	75	++	++	++	+	-	-
	Collodion	Normal	-	-	-	-	-	-

Solution of antigen obtained from myxomatous skin of rabbits.

See text for preparation of antigen-collodion mixtures.

Antiserum 75 obtained from rabbit immunized with non-infectious extract of myxomatous skin.

* Dilution 1:8.

quently cleared of the small amount of insoluble material that appeared during treatment. The precipitin titer of the solution was 1:32 before heating and 0 after heating when tested with optimal amounts of anti-soluble-substance serum number 75; a 1:256 dilution of the antigen either unheated or heated fixed complement in the presence of the same serum. Collodion particles of about the size of elementary bodies of vaccinia (Shedlovsky and Smadel, 1940) were added to solutions of unheated and of heated A antigen in the following proportions: 1 ml. of solution of antigen, 3 ml. of saline and 3.5 ml. of buffer (.002 M, pH 7.2) solutions, and, finally, .5 ml. of a heavy stock suspension of graded collodion

particles. The faintly opalescent suspension which was stable after storage overnight at 5°C. was employed in the type of agglutination test usually used for elementary bodies. Control precipitin tests with the same dilutions of antigen (without collodion) and antiserum were run concurrently. It is apparent from the results summarized in table 5 that the presence of collodion particles did not appreciably enhance the flocculation titer of antiserum number 75 with unheated A antigen. On the other hand, the flocculation obtained with the solution of heated A antigen that contained collodion particles was almost as great as that exhibited by the unheated antigen, but the solution of heated antigen, without collodion particles, failed to give a visible reaction. These observations indicate that heated soluble antigen A of myxoma which has been adsorbed on collodion particles can be flocculated by specific antibody.

DISCUSSION

The present experiments indicate that the soluble antigens of infectious myxomatosis occur in chorioallantoic membranes infected with the virus of this disease. Thus, myxoma apparently conforms to the general pattern of viruses which have associated soluble antigens in that its specific soluble precipitable substances are found in infected tissue of different susceptible hosts. During the course of this work Hoffstadt and Pilcher (1939) reported the results of an investigation that had a similar objective. These workers noted a specific precipitate when filtrates of myxomatous membranes were tested with fibro-myxoma serum prepared in rabbits; our findings confirm and extend their observations. A comparison of the results of experiments of Hoffstadt and Pilcher (1939) and of ourselves dealing with the preparation of suspensions of elementary bodies from infected eggs and with their properties are in essential agreement. The data from the two laboratories differ regarding agglutinability of such suspensions of virus by immune serum but this can undoubtedly be accounted for on the basis of the technique employed.

Hyde (1939) recently reported that fixation of complement occurred in mixtures containing preparations of myxomatous

tissue from rabbits and fibro-myxoma antiserum; the method of preparing the tissue for testing was not described in detail but the material was apparently a partially clarified crude suspension which may be assumed to have contained both virus and soluble antigen. The present experiments indicate that both washed virus and solutions of antigen are capable of fixing complement in the presence of myxoma immune and anti-soluble-substance sera. It was surprising to find that solutions of antigen which have been degraded by heat to the stage where they are no longer capable of precipitating with specific antibody can still fix complement in as high a dilution as before heating. It has been observed that dermal filtrate containing the labile soluble antigen of vaccinia (L) no longer precipitates or fixes complement with its antibody after similar thermal inactivation (Craigie and Wishart, 1936).

The flocculation of collodion particles coated with heated A antigen by anti-soluble-substance serum provides a basis for explaining the earlier puzzling observation (Rivers, Ward and Smadel, 1939) that elementary bodies of myxoma which were agglutinated by anti-soluble-substance serum retained an appreciable amount of their agglutinability after heating. That there is a heat-stable agglutinin of myxoma which differs from degraded soluble antigen may be deduced from evidence which has been reported (Rivers, Ward and Smadel, 1939). Nevertheless, the rôle of degraded soluble antigen as one of the agglutinogens of heated elementary bodies of myxoma must be kept in mind in future studies.

SUMMARY

Elementary bodies and specific soluble antigens can be obtained from the chorioallantoic membranes of developing chick embryos infected with the virus of infectious myxomatosis.

The A and B soluble antigens of myxoma which lose their specific precipitability on heating retain their power to fix complement in the presence of specific antibody.

Collodion particles coated with heated A antigen are flocculated by anti-soluble-substance serum.

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THE LS-ANTIGEN OF VACCINIA

I. INHIBITION OF L- AND S-ANTIBODIES BY SUBSTANCES IN TREATED VACCINE DERMAL FILTRATE*

By JOSEPH E. SMADEL, M.D., AND THOMAS M. RIVERS, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

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A relationship between the heat-labile (L) and heat-stable (S) soluble antigens of vaccinia has been shown to exist by several workers. Craigie and Wishart (1) originally regarded the two antigens as occurring in the form of a complex which elicited two separate antibodies in animals. They were led to this conclusion because both antigens regularly are found in equal amounts in filtrates of infected tissue and because absorption with either specific antibody removes both serologically active substances. Parker (2) observed that under certain conditions different results were obtained, *e.g.*, a solution containing both precipitable substances when treated with S-antibody lost its S-antigen but retained some L-antigen. Craigie and Wishart (3), at about this time, made similar observations; they reaffirmed that L-antigen ordinarily occurs in a state of combination with S-antigen but reported that with prolonged storage in the cold "this combination may dissociate into separate L and S fractions prior to ultimate inactivation of the L antigen." The data of Parker and of Craigie and Wishart that suggested dissociation of L- and S-antigens have not been duplicated in our laboratory. This failure we believe indicates that conditions for successful repetition of such experiments occur infrequently. That they do occur occasionally we have no doubt, for, as will be shown in a subsequent paper (4), a solution with L-activity only can be prepared from pure LS-antigen by enzymatic digestion.

In our experience, L-antigen has always been encountered in association with S-antigen except under the special circumstances just mentioned. This was true even though at various times during the past five years we have thought of the L-reacting substance as a protein, a carbohydrate, a fat, or a polypeptide and have attempted to isolate such an hypothetical material from dermal filtrates by appropriate methods. When it became evident that the methods employed were inadequate to procure preparations of L-antigen which were free of S-antigen for comparison with the relatively pure S-protein of Parker

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and Rivers (5), other means were sought for the study of the relationship of these two antigens of vaccinia.

The inhibition technique used in serological work assisted in establishing the existence of partially degraded forms of the A and B antigens of infectious myxomatosis of rabbits (6). These two protein-like antigens readily lose their specific precipitability on gentle heating or even on storage. Solutions of A-antigen, which are no longer capable of precipitating with A-antibody, still combine with it as demonstrated by the inhibition technique. Solutions of degraded B-antigen behave similarly. Inhibition by the non-precipitable A- and B-antigens is specific, *i.e.*, each antibody combines only with the degraded form of its own antigen. In view of the results obtained with myxoma antigens, inhibition of the L- and S-antibodies of vaccinia was investigated with the hope of demonstrating serologically degraded forms of the L- and S-antigens. It was thought that experimentation with such degraded substances might provide data which would help in interpreting the interrelationship of the two native antigens. The results of these investigations are reported in this paper.

Materials and Methods

Source of Soluble Antigens.—Dermal filtrate containing the soluble antigens of vaccinia was obtained from dermal pulp of rabbits which had been cutaneously infected 3 days previously with the C.L. strain of vaccine virus (1). The pulp from each rabbit was suspended in 30 to 40 cc. of a 1:50 dilution of disodium phosphate-citric acid buffer solution, pH 7.2. The suspension was freed of gross particles and of essentially all of the elementary bodies of vaccinia by differential centrifugation, and, finally, filtered through a Seitz pad to remove all residual virus. These filtrates were stored at 3°C. until used.

Antisera.—L-antiserum was prepared by absorbing S-antibody from hyperimmune antivaccinal serum. Vaccine virus immune rabbits which had been given a course of intravenous injections of active elementary bodies of vaccinia provided the hyperimmune serum; absorption of S-antibody was carried out with proper amounts of heated dermal filtrate. S-antisera were obtained from non-immune rabbits after several courses of injections of heat-inactivated elementary bodies.

Precipitin Tests.—0.25 cc. volumes of serial dilutions of solution of antigen were prepared in physiological saline solution buffered at pH 7.2 and mixed with 0.25 cc. amounts of an appropriate dilution of antiserum. Readings were made after incubation overnight in closed tubes held at 50°C.

EXPERIMENTAL

General Properties of L-Antigen.—Only a few procedures have been found practicable for the concentration of L-antigen from filtrates of dermal pulp obtained from rabbits infected with the virus of vaccinia. These methods are equally efficacious for the concentration of S-antigen. Craigie and Wishart (1) have observed that both serologically active substances can be obtained

from virus-free dermal filtrate by bringing the pH of the solution to 4.5 and then redissolving the insoluble material thus obtained at pH 6.6. In our hands this method has proved the simplest and the most satisfactory for obtaining solutions containing large amounts of L- and S-antigens from which have been eliminated appreciable amounts of serologically inert material. Moderate variations in the pH ranges employed for precipitating and for redissolving the antigens still result in final solutions with equal amounts of the two serologically active substances; in some instances, however, the recovery of antigenic material is less complete than it is with Craigie and Wishart's technique. Bringing the pH to below 4.0 results in a loss of precipitability of L with its antibody, but S-activity is not appreciably reduced even at pH 1.8. On the alkaline side of neutrality both serological substances retain their activity for many days in the cold at pH 9.0. Furthermore, the L-antigen is unaffected by short exposure to borate buffer of pH 11.0, while the S-antigen is inactivated in $N/20$ NaOH only after minutes or hours depending on whether the procedure is carried out at 56°C. or at 3°C. Treatment with $N/20$ NaOH rapidly destroys the precipitability of L-antigen.

Under the proper conditions, precipitation of concentrated unheated dermal filtrate with ammonium sulfate yields equal quantities of L- and S-antigen in the globulin fraction. L-activity is generally lost during fractional precipitation with ammonium sulfate unless filtrates which have been concentrated 10 to 20 times by evaporation are used as starting material; preliminary concentrations of dermal filtrate is unnecessary, however, if the objective is purification of S-antigen. Evaporation of dermal filtrate through cellophane sausage casings suspended in an air stream at room temperature provides a ready means of concentrating the soluble antigens, but fails to eliminate any significant amount of serologically inert substances. On the other hand, drying large volumes of dermal filtrate from the frozen state renders some of the inert material insoluble on subsequent resuspension. Although laborious, this procedure can be employed for concentration of the S-antigen, but it is often unsatisfactory for work with L-antigen since temporary thawing during the process may be followed by partial or complete loss of L-precipitability.

The boiling of solutions containing antigens of vaccinia at pH values near neutrality with subsequent removal of coagulated protein has been found by several workers (5, 7, 8) to be of assistance in the partial purification of solutions of the heat-stable antigen, but obviously this procedure has no place in experiments designed for the purification of the heat-labile material. Similarly, treatment of dermal filtrate with large volumes of cold alcohol results in complete disappearance of the substance capable of precipitating with L-antibody even though the stable antigen can be recovered from the alcohol insoluble fraction as has been demonstrated by others (5, 8).

The L-antigen of vaccinia may be regarded, therefore, as having certain

properties in common with S-antigen. Both are insoluble in the neighborhood of pH 4.5 and soluble in the region of pH 6.5, and both are found in the globulin fraction when separated from concentrated dermal filtrate by partial saturation with ammonium sulfate. The heat-labile antigen, however, readily loses its specific precipitability under conditions which do not alter the heat-stable antigen, *viz.*, extremes of pH, treatment with alcohol, and, in certain instances, drying from the frozen state or precipitation with ammonium sulfate. The common solubilities of the two antigens give no clue to their nature, since the data might equally well suggest that the antigens are parts of a single protein molecule, or that they are similar protein substances, or that L is a non-protein material associated with the S-protein molecule.

Inhibition of L-Antibody

Solutions containing both L- and S-precipitable substances in high concentration showed no change in physical appearance when heated at 56°C. for 1 hour. This led us to think that the L-antigen, which was no longer demonstrable by the usual precipitation technique, might still be present in these heated solutions in an altered form which was unable to precipitate with L-antibody. It seemed possible, furthermore, that such an altered antigen might inhibit L-antibody, and, indeed, this was found to be the case as shown by the following experiment.

Experiment 1.—1500 cc. of dermal filtrate were placed in cellophane tubes and concentrated to 100 cc. by evaporation. The material was dialyzed against running water overnight, and while still in the original cellophane tube was again concentrated by evaporation to 50 cc. This solution reacted in a dilution of 1:1024 with optimal amounts of both L- and S-antibodies. Globulin material was obtained from the concentrate by fractional precipitation with ammonium sulfate. 50 cc. of a solution of the reprecipitated globulin material were treated with 5 cc. of standard citric acid-disodium phosphate buffer, pH 4.5. Most of the material insoluble under these conditions redissolved when taken up in 20 cc. of diluted buffer solution, pH 6.6. This solution, containing globulin material insoluble at pH 4.5 and soluble at pH 6.6, had as much L- and S-activity as did the original concentrated dermal filtrate. After heating at 56°C. for 1 hour the solution no longer precipitated with L-antiserum but its titer with S-antiserum was not reduced. One cc. of the heated solution was added to an equal amount of undiluted L-antiserum; the mixture was diluted with 4 cc. of saline solution, incubated at 56°C. for 1 hour, and stored at 3°C. for 24 hours. No precipitate appeared. This treated mixture, which should have contained sufficient L-antibody to react strongly with unheated L-antigen, failed to form a precipitate when added to serial dilutions of unheated dermal filtrate and incubated under the usual conditions. The inhibition experiment was repeated with identical results when portions of the same solution of antigen which had been heated at 60°C., 80°C., and 90°C. for 1 hour were used.

In the experiment just described L-antigen in the concentrated globulin material from dermal filtrate was affected in the usual manner by heating, *i.e.*, it no longer precipitated with L-antibody. A substance was present, however, in the heated solutions which was capable of inhibiting in some way the flocculation of L-antibody with unheated L-antigen. Since the partially purified material employed in the experiment was highly concentrated, it seemed desirable to repeat the inhibition procedure with a more dilute solution. For this purpose crude dermal filtrate prepared according to routine was used.

Experiment 2.—Dermal filtrate, pool 6, which precipitated in a dilution of 1:128 in the presence of optimal amounts of either L- or S-antibody was heated in a closed flask in a water bath at 80°C. for 1 hour. The heated filtrate which no longer pre-

TABLE I
Inhibition of L-Antibody by Heated Dermal Filtrate

Antigen	Treatment of L-antiserum 6814*	Dilution of antigen					
		1:8	1:16	1:32	1:64	1:128	1:256
Dermal filtrate 6 (untreated)	Control	+++	++++	++++	+++	+	—
	1 volume heated vac- cinia dermal filtrate 6	++	++++	+++	±	—	—
	3 volumes heated vac- cinia dermal filtrate 6	—	—	±	—	—	—
	5 volumes heated vac- cinia dermal filtrate 6	—	—	—	—	—	—
	5 volumes heated myx- oma dermal filtrate	++++	++++	++++	+++	±	—

* Dilution of antiserum was 1:8 in each test.

cipitated with L-serum was added in varying amounts to 0.5 cc. portions of undiluted L-antiserum. Sufficient saline solution was added to each mixture to bring the final dilution of serum to 1:8 and the mixtures were incubated at 56°C. for 1 hour. Samples of the treated serum, together with a control serum diluted only with saline, were tested for their capacity to precipitate with serial dilutions of unheated dermal filtrate, pool 6. The results, summarized in Table I, show that the addition of an equal volume of heated filtrate to L-antiserum appreciably decreased the power of the serum to react with unheated filtrate. Furthermore, no precipitation occurred when the serum mixtures containing 3 and 5 volumes, respectively, of heat-inactivated filtrate were tested against solutions known to possess active L-antigen.

The data obtained in Experiments 1 and 2 indicate that crude or partially purified solutions containing either small or large amounts of L- and S-substances lose their capacity to precipitate with L-antibody after heating, but are, nevertheless, still able to inhibit L-antibody.

Inhibition of anti-soluble substance antibodies of vaccinia has been ob-

served by others. Craigie and Wishart (1) found that certain heated crude dermal filtrates employed for absorption studies with antivaccinal serum contained inhibiting substances which were carried along with the absorbed serum and subsequently interfered with the demonstration of residual antibodies. These authors regarded the inhibitory substances as non-specific in character and found that fractionation of the filtrates resulted in their elimination. Salaman (9) likewise observed reductions in precipitin and agglutinin titers following absorption of antiserum with large amounts of dermal filtrate; he regarded these effects as examples of the familiar inhibition which occurs in the presence of an excess of antigen and thought that they should not be called non-specific. Since the L-antigen had been rendered non-precipitable by heating, the inhibition of L-antibody observed in Experiments 1 and 2 was certainly not dependent upon the presence of an excess of precipitable L-antigen. Moreover, that the inhibition was serologically specific was demonstrated in the following manner.

Experiment 3.—Dermal filtrate was prepared from rabbits cutaneously infected with the virus of myxomatosis by a technique essentially identical with that employed for rabbits infected with vaccinia. The filtrate which was rich in the A and B soluble antigens of myxoma was heated at 56°C. for 1 hour and then mixed with antivaccinal serum. The mixtures were tested for L-precipitins of vaccinia in the usual manner. As indicated by the results summarized in Table I, no inhibition of L-antibody occurred even when 5 volumes of the myxomatous filtrate were added to the L-antiserum. It has already been recorded that vaccine dermal filtrate fails to inhibit the antisoluble substance antibodies of myxomatosis (6).

Heated dermal filtrate appeared to be capable of specifically inhibiting L-antibody of vaccinia. It will be recalled that L-antisera are regularly prepared by absorbing hyperimmune antivaccinal serum with heated dermal filtrate; this absorption procedure when carried out under the proper conditions removes S-antibody and leaves L-antibody. This apparent contradiction was shown to depend on a quantitative factor; a much smaller amount of heated filtrate was generally needed to absorb S-antibody from immune serum than was necessary to obtain demonstrable inhibition of L-antibody.

Experiment 4.—Unabsorbed hyperimmune serum, number 6814, reacted in a dilution of 1:32 with an optimal amount of unheated filtrate, pool 6, and in a dilution of 1:4 with heated filtrate. Complete removal of S-antibody without a detectable reduction in the quantity of L-antibody resulted when the hyperimmune serum was absorbed with an equal volume of a 1:8 dilution of dermal filtrate, pool 6, which had been heated at 80°C. for 1 hour. L-precipitins, however, were no longer demonstrable in this antiserum after it was treated with an equal volume of four-times concentrated solution of heated filtrate, pool 6. The filtrate had been concentrated by evaporation in a cellophane bag. Results of this experiment are summarized in Table II.

The material having the property of inhibiting L-antibody in the experiments so far presented always occurred in association with S-antigen. It seemed desirable, therefore, to prepare purified S-antigen by the method of Parker and Rivers (5) and to test the power of this substance to block or inhibit L-antibody.

Experiment 5.—625 cc. of dermal filtrate, pool 7, having a titer of 1:128 with optimal dilutions of L- and S-antisera were boiled and subsequently the globulin fraction was salted out with ammonium sulfate. The redissolved globulin fraction was precipitated with alcohol, redissolved in water, precipitated at pH 4.6 with citric acid-disodium phosphate buffer solution, and, finally, redissolved in dilute citric acid-disodium phosphate buffer solution, pH 8.0. This solution was boiled, the pH was then changed to

TABLE II

Preparation of L-Serum by Absorption of Immune Serum with Heated Dermal Filtrate and Inhibition of L-Antibody with the Same Material

Hyperimmune serum 6814	Antigen: Dermal filtrate 6 diluted 1:16	Dilution of serum						
		1:2	1:4	1:8	1:16	1:32	1:64	1:128
Untreated	Unheated	++++	++++	++++	++++	++++	?	—
	Heated	++++	++	—	—	—	—	—
Absorbed*	Unheated	++++	++++	++++	++++	++++	?	—
	Heated	—	—	—	—	—	—	—
Inhibited†	Unheated	—	—	—	—	—	—	—

* Absorbed serum was prepared by treating 1.5 cc. of serum 6814 with 1.5 cc. of a 1:8 dilution of heated dermal filtrate 6.

† Inhibited serum was prepared by treating 6814 with an equal volume of 4 × concentrated solution of heated dermal filtrate 6.

6.0 by the addition of sodium hydroxide, the solution was again boiled, and then dialyzed free of salts. 0.25 cc. of a 1:1024 dilution of this final solution gave a precipitate with optimal amounts of S-antibody. A portion of the 41 cc. of final solution was dried from the frozen state and the residue was weighed; the solution before drying was shown to have contained 5.1 mg. of dry material per cc. Hence, on the basis of precipitin titer and dry weight, it was estimated that 1 part in 800,000 was sufficient to give a specific reaction with S-antiserum. The relative purity of the final preparation appeared to be of the same order as that obtained by Parker and Rivers. Portions of the final preparation of S-antigen as well as samples of different fractions obtained during the process of purification were tested in the usual way for their inhibitory effect on 0.5 cc. volumes of L-antiserum, number 6814. The results are summarized in Table III.

The results summarized in Table III show that the substance in crude vaccine dermal filtrate capable of inhibiting L-antibody was also present in materials

obtained at intermediate stages and in the final stage of the purification of S-antigen. These data indicate that L-inhibitor, like the precipitable form of L-antigen, is closely associated with S-antigen.

Inhibition of S-Antibody

Earlier observations (10) indicated that S-antigen which has been heated with dilute alkali is not precipitated by its antibody. The capacity of such a degraded antigen to combine with S-antibody was investigated by means of the inhibition technique. The following protocol illustrates the results obtained in a typical experiment.

Experiment 6.—40 mg. of the final dried preparation of S-antigen from Experiment 5 were dissolved in 8 cc. of N/20 NaOH. The solution remained clear after heating

TABLE III
Inhibition of L-Antibody by a Preparation of S-Antigen

Antigen	Fraction prepared from heated dermal filtrate	L-antiserum treated with fraction	Dilution of untreated dermal filtrate				
			1:8	1:16	1:32	1:64	1:128
Dermal filtrate		None	+++	++++	++++	+++	+
	Globulin fraction	1 volume	—	—	—	—	—
	Alcohol insoluble fraction of globulin	1 "	—	—	—	—	—
	Final solution of S-antigen	1 "	++	++	+	—	—
	" " "	2 volumes	—	±	—	—	—

Dilution of L-antiserum was 1:6 in all tests. Inhibited sera were obtained by adding 1 or 2 volumes of test material to 0.5 cc. amounts of L-antiserum. S-antigen titer of each test material was 1:1024. Final solution of S contained 5 mg. per cc.

at 56°C. for 90 minutes. A faint opalescence which appeared when the treated solution was brought to pH 7.2 with N/1 HCl was eliminated by ultracentrifugation at 30,000 R.P.M.; the small amount of sediment thus obtained was discarded. Serial dilutions of the clear supernatant fluid which contained practically all of the degraded S-antigen did not form precipitates when incubated with optimal amounts of S-antibody. Varying quantities of the undiluted solution of treated antigen were mixed with 0.4 cc. volumes of S-antiserum, number 274, and with 0.3 cc. volumes of L-antiserum, number 6814; sufficient saline solution was added to each mixture to bring the final concentrations of antisera to their optimal dilutions. The mixtures, all of which remained clear after incubation at 56°C. for 1 hour, were tested for demonstrable precipitins against L- and S-antigens by the usual methods. The results are summarized in Table IV.

The data presented in Table IV indicate that S-antigen of vaccinia which has been degraded by heat and alkali to a stage where it no longer precipitates with

its antibody is still able to combine with this antibody. For example, 0.5 mg. of treated antigen completely inhibited the antibody in 0.4 cc. of undiluted S-antiserum. It is also evident that this preparation of degraded antigen had only a slight capacity to inhibit L-antibody. That the property of the degraded S-antigen to inhibit L-antibody was indeed less than that of the purified precipitable S-antigen from which it was derived may be seen by comparing the data given in the protocols of Experiments 5 and 6; for instance, 2 to 3 times as much of the alkali-treated material was needed to produce the slight inhibitory effect on L-antibody observed in Experiment 5 where undegraded S-antigen was used.

TABLE IV
Inhibition of S-Antibody

Antigen	Antiserum	Treatment of antiserum with degraded S	Dilution of antigen				
			1:8	1:16	1:32	1:64	1:128
Dermal filtrate	S (diluted 1:4)	mg.					
		None	+++~	++++	++++	++++	++
		0.125	+++	++++	++++	++++	++
		0.25	?	+++	++++	+	-
		0.50	-	-	-	-	-
		0.75	-	-	-	-	-
		1.00	-	-	-	-	-
	L (diluted 1:6)	None	+++	++++	++++	++++	++
		1.00	++++	++++	++++	+++	+
		7.50	+++	++	++	-	-

0.4 cc. volumes of S-antiserum and 0.3 cc. volumes of L-antiserum were treated with the designated amount of S-antigen which had been heated at 56°C. for 90 minutes in the presence of N/20 NaOH.

Inhibition of S-antibody can also be demonstrated by adding the antibody directly to mixtures of native and degraded S-antigen. In this type of experiment, however, the latter material must be present in large amounts in comparison to the former. This is illustrated by the data presented in Table V which summarizes the results obtained when alkali and heat-treated S-antigen from Experiment 6 was added in increasing amounts to 0.4 cc. volumes of unheated dermal filtrate, after which serial dilutions of the mixtures were prepared and incubated with optimal amounts of L- or S-antibody. Results of the titrations made with the mixture containing 0.1 mg. of degraded S-antigen were comparable to the controls. The presence of 1.0 mg. of the non-precipitable or degraded antigen prevented flocculation of the native S-antigen only in the lowest dilutions of the titrations with S-antibody, while 5.0 mg. completely blocked precipitation of native S-antigen with its antibody through-

out the range of dilutions. In none of the mixtures was the reaction with L-antibody appreciably altered.

On several occasions S-inhibitor has been encountered in preparations of vaccine dermal filtrate that were not subjected to alkaline treatment. In these experiments the filtrates were concentrated 10 to 20 times before boiling. The technique for purifying S-antigen was originally applied to crude dermal filtrates (5). In order to avoid working with large volumes, we first concentrated the crude filtrates by evaporation in cellophane tubes. The results obtained when the purification procedure was applied to these concentrates were disappointing in that yield of S-antigen was negligible as determined by pre-

TABLE V
Inhibition of S-Antibody—Continued

Antigen mixture		Antiserum	Dilution of original dermal filtrate			
Dermal filtrate	Degraded S-antigen		1:8	1:16	1:32	1:64
cc.	mg.					
0.4	0.1	L	++++	++++	++++	++
		S	++++	++++	++++	+++
0.4	1.0	L	++++	++++	+++	++
		S	—	++++	++++	+++
0.4	2.0	L	++++	++++	+++	++
		S	—	—	—	++
0.4	5.0	L	++++	++++	+++	+
		S	—	—	—	—

Mixtures of dermal filtrate and degraded S-antigen were incubated at 37°C. for ½ hour and then diluted serially. Constant amounts of L-antiserum 6814 diluted 1:6 and of S-antiserum 274 diluted 1:4 were added and the titration results were read after incubation overnight at 50°C.

cipitin tests. The failure to demonstrate the expected amounts of S-antigen in the solutions was found to be dependent upon the presence of an inhibitor which could be removed, leaving behind precipitable S-antigen. The results of this type of work are illustrated by the following experiment.

Experiment 7.—960 cc. of dermal filtrate, pool 8, were dialyzed in cellophane tubes against running water and then concentrated to 90 cc. in the same tubes by evaporation in a stream of air. The clear solution became opalescent after gentle boiling for 5 minutes, and the small amount of precipitate that formed was removed by centrifugation. The globulin fraction obtained by precipitation with ammonium sulfate was redissolved in water, dialyzed free of sulfate ions, and brought back to a volume of 90 cc. The original crude dermal filtrate in a dilution of 1:64 precipitated with L- and S-antisera, but the concentrated solution of heated globulin gave only a slight precipitate in dilutions of 1:8 to 1:32 with S-antiserum. A portion of the solution

of globulin was fractionated by the method of Craigie and Wishart (1) for the concentration of LS-antigen. Only about half of the voluminous white precipitate obtained at pH 4.5 redissolved when it was suspended in buffer solution, pH 6.6. The solution containing the material which dissolved at pH 6.6 had a precipitin titer of 1:128 when tested with S-antiserum. The material which failed to dissolve at pH 6.6 was soluble in dilute buffer solution, pH 9.0, and the solution remained clear when brought back to pH 7.0. While this solution did not precipitate in dilutions of 1:4 to 1:512 in the presence of an optimal amount of S-antibody, it was found to have a marked inhibiting effect on S-antibody. S-antiserum treated with 3 volumes of the solution would not precipitate with native S-antigen. On the other hand, L-antiserum was not appreciably inhibited by 5 volumes of the solution.

The results obtained in Experiment 7 throw some light on the work of Craigie and Wishart (1). For example, it was shown that under certain conditions the heating of dermal filtrate, even in the pH range near neutral reactions, may result in the formation of S-inhibitor. Furthermore, it was found that S-inhibitor can be separated from precipitable S-antigen by means of the different solubilities of the two substances at several pH values.

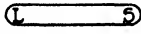
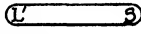
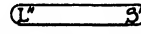
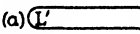
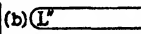
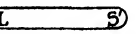
DISCUSSION

The results of the foregoing experiments may be briefly summarized as follows. Dermal filtrate prepared from the skin of rabbits infected with the virus of vaccinia contains the heat-labile (L) and heat-stable (S) antigens of vaccinia which can be demonstrated by the precipitation technique. Gentle heating destroys the precipitability of L-antigen but leaves in solution a substance capable of inhibiting L-antibody. This L-inhibitor, like precipitable L-antigen, is closely associated with S-antigen and cannot readily be separated from it. Preparations of S-antigen can also be degraded by any of several methods to a stage where precipitation with S-antibody does not occur; however, inhibition of S-antibody with this material is easily demonstrated. Degraded S-antigen, in contrast to precipitable S-antigen, has little power to inhibit L-antibody. The solubilities of S-inhibitor are different from those of S-antigen and L-inhibitor.

The immediate objective of the present experiments, *viz.*, the demonstration of degraded forms of L- and S-antigens of vaccinia which are capable of inhibiting their specific antibodies, was accomplished. The studies were less successful, however, in regard to their ultimate objective which was the elucidation of the relationship between L- and S-antigens. Nevertheless, the observations just reported may serve as the basis for several hypotheses on the relationship of these two serologically active substances.

One of the simplest of the hypotheses is as follows: The native antigen present in infected tissue is L, or the antecedent substance from which it is derived. L is readily degraded during manipulation and storage to a slightly modified

material, S, which is still capable of eliciting a specific antibody in animals and which combines with L-antibody as demonstrated by inhibition tests. S-antigen can be further degraded to a stage where it inhibits but fails to precipitate with its antibody; and, in this form it loses most of its ability to combine with L-antibody. A graphic representation of the various stages of degradation according to this scheme is presented in Text-fig. 1. There are

Hypothesis	Native materials in dermal filtrate	Effect of heat	Effect of heat and alkali
I	L Precipitates with L-antibody	S Inhibits L-antibody Precipitates with S-antibody	S' No inhibition of L-antibody No precipitation with S-antibody Inhibits S-antibody
II	L Precipitates with L-antibody S Precipitates with S-antibody	L' Inhibits L-antibody S Precipitates with S-antibody	L'' No inhibition of L-antibody S' Inhibits S-antibody
III	 Precipitates with L-antibody Precipitates with S-antibody	 Inhibits L-antibody Precipitates with S-antibody	 No inhibition of L-antibody No precipitation with S-antibody Inhibition of S-antibody
	Other theoretical combinations		
	(a)  Precipitates with neither antibody Inhibits both antibodies	(b)  Neither precipi- tates nor inhibits	(c)  Precipitates with L-antibody No precipitation with S-antibody Inhibits S-antibody

TEXT-FIG. 1

several serious objections to this hypothesis. No reasonable explanation is at hand for the regular occurrence of equal amounts of L- and S-antigen in preparations from infected tissue. Moreover, it should be possible, if the hypothesis were correct, to demonstrate a simultaneous increase in S along with a decrease in L during the various procedures which render L-antigen non-precipitable.

A second hypothesis might begin with the assumption that L- and S-antigens are separate protein molecules which are distinguishable by serological means but not by ordinary physical and chemical procedures. According to this idea each of the antigens could be degraded independently, but, since L is more

easily affected than S, the labile antigen would generally be denatured one step further than the stable one (Text-fig. 1). The similar physicochemical nature of the native substances might also be considered to be characteristic of the mildly degraded antigens; thus, a separation of L-inhibitor and S-antigen would be difficult. This hypothesis, like the first, affords no explanation for the uniform ratio of L- and S-antigens in crude filtrates. Furthermore, it is hard to reconcile it with the almost invariable results obtained in absorption experiments, namely, the removal of both antigens on the addition of either antibody.

A third hypothesis deals with an LS-complex similar to that postulated by Craigie and Wishart (1). No assumptions need be made about the chemical nature of the L-part of this complex or about the type of union between it and the protein-like S-part. As in the second hypothesis, the L-reacting substance would be considered to be degraded more readily than the S under ordinary conditions, and complexes might be formed of L'S and L''S' on treatment with heat or heat and alkali, respectively (Text-fig. 1). In such a scheme the first degradation product, *e.g.*, L' would inhibit L-antibody but not be precipitated by it, while L'' would have lost most of its capacity to inhibit L-antibody. Similarly, stages in the degradation of the S-portion of the complex could be postulated. Additional complexes with various combinations of native and degraded stages of the L- and S-parts might be considered; several of these are represented in Text-fig. 1, (a), (b), (c). The objections raised to the first two hypotheses are not valid for the last one. The unusual results of occasional absorption experiments reported by Parker (2) and Craigie and Wishart (3) can be explained by this hypothesis if one assumes that under certain rare conditions a portion of the LS-complex in dermal filtrate is degraded to a form represented as LS' (Text-fig. 1, (c)). A mixture of this complex and native LS when treated with S-antibody could result in the precipitation of LS, leaving in solution LS' combined with S-antibody. Such a soluble antigen-antibody combination might then be precipitated on the addition of L-antibody.

At the time these experiments were completed we were inclined to accept the third hypothesis as the one which more nearly explained the available data on the relationship of the L- and S-antigens of vaccinia. Work reported in accompanying and subsequent papers (4, 11) has done much to strengthen our belief that this hypothesis is substantially correct.

CONCLUSION

Experimental data are presented which may be interpreted as follows. The heat-labile (L) and heat-stable (S) antigens of vaccinia occur in nature as a complex consisting of a single substance with two serologically active parts, each of which may be degraded independently of the other.

We wish to express our appreciation to Dr. Kenneth Goodner for his advice.

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THE LS-ANTIGEN OF VACCINIA

II. ISOLATION OF A SINGLE SUBSTANCE CONTAINING BOTH L- AND S-ACTIVITY

By THEODORE SHEDLOVSKY, Ph.D., AND JOSEPH E. SMADEL, M.D.

(From the Laboratories and the Hospital of The Rockefeller Institute for Medical Research)

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The intimate association of the heat-labile (L) and heat-stable (S) soluble antigens of vaccinia was shown by the work of Craigie and Wishart (1). Moreover, observations on the degraded forms of these antigens, reported in the previous paper (2), further indicated the close relationship of these two antigens. The fact that the L-antigen is readily denatured by a number of procedures which are ordinarily used for purification of immunologically active substances has delayed the acquisition of knowledge concerning its nature. On the other hand, the heat-stable antigen has been studied in considerable detail (3, 4) and has been shown to be a protein substance. Although the results of several experiments (4, 5) have been interpreted as indicating that L-antigen may be obtained free of the S-antigen, these experiments are also subject to a different interpretation (2). The work reported here was undertaken in the hope of obtaining pure L-antigen in sufficient amounts to study its physical and immunochemical properties. As will be shown, the L-antigen is not found free from the S-reacting substance under ordinary conditions. Indeed, the results indicate that the L- and S-immunological activities reside in a single native protein molecule. A method for isolating pure LS-antigen of vaccinia from virus-free filtrates of infected rabbit skin will be given in this communication together with observations on the physical and immunological properties of the native substance and of some of its degraded forms.

Materials and Methods

Vaccine Dermal Filtrate.—Dermal filtrate was available in large quantities as a by-product obtained from the preparation of washed elementary bodies of vaccinia which were used for other studies. The technique of Craigie (6) was employed without significant modification. Dermal pulp from each cutaneously infected rabbit was suspended in 30 cc. of a 1:50 dilution of standard phosphate buffer solution pH 7.2 and the solution which remained after removal of tissue particles and elementary bodies by means of differential centrifugation was filtered immediately through a Seitz pad. Bacteriologically sterile virus-free filtrates were stored at 3°C. until 800 to 1200 cc. had been accumulated, usually a period of a month.

Antiserum.—L-antiserum used throughout this portion of the work was prepared from pooled sera obtained by bleeding vaccine virus immune rabbits which had been hyperimmunized with washed active elementary bodies of vaccinia; the serum was absorbed free of S-antibody with heated crude dermal filtrate. S-antisera were obtained from rabbits which had been immunized with a non-infectious solution of purified S-antigen (4). Precautions were taken to prevent these non-immune animals from becoming accidentally infected with vaccinia during the period of immunization. Our experience, like that of Parker, indicated that solutions of S-antigen prepared in this manner elicit a poor antibody response in rabbits and in order to obtain potent sera several courses of treatment were given. For the final course of immunization, washed and graded collodion particles were added to the solution of S-antigen and the mixture was injected intravenously.

Serological Methods.—The serological techniques employed in this work have been reviewed in a previous paper (2).

Electrophoretic Technique.—Electrophoretic experiments were carried out in the Tiselius apparatus with the schlieren-scanning optical system (7) the details of which have been discussed elsewhere. The temperature of the thermostat containing the cell was regulated at 1°C., but all the values of mobility given in this paper have been corrected to 0°C.

EXPERIMENTAL

Observations on Concentrated Whole Dermal Filtrate.—The results obtained in studies in which the electrophoretic technique was applied to concentrated dermal filtrate, both before and after heating, are illustrated in the following experiments.

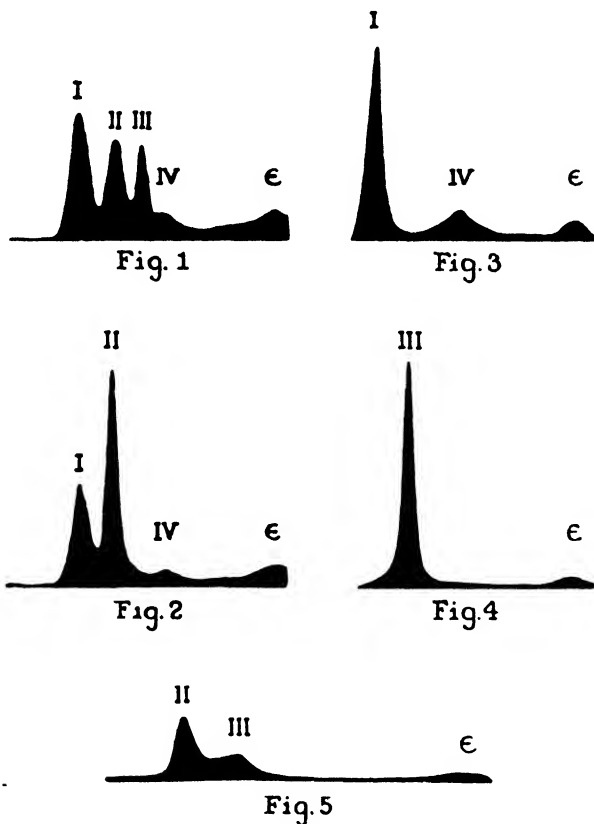
Experiment 1.—One liter of dermal filtrate was concentrated to a volume of 100 cc. by means of evaporation through a cellophane sausage casing placed in an air stream at room temperature for 24 hours. The L- and S-titers of this concentrate were 1:1000. A portion of the material was dialyzed against 0.05 M lithium-veronal buffer solution at pH 7.9.¹ Upon electrophoresis this dialyzed material was found to display four boundaries, all of which corresponded to negatively charged substances at this value of pH. A reproduction of the electrophoretic pattern obtained is shown in Fig. 1. The mobilities of the four components, which were designated I to IV, were 6.7×10^{-5} , 5.4×10^{-5} , 4.4×10^{-5} , and 3.4×10^{-5} cm./sec. per volt/cm., respectively. In Fig. 1, as in subsequent figures, the first peak on the right, ϵ , does not correspond to any component, but is due to a gradient of buffer salts.

Experiment 2.—A portion of the same material was heated at 70°C. for ½ hour in a closed vessel (a procedure which inactivates L without destroying S) and an electro-

¹ This solution contained diethylbarbituric acid, its lithium salt, and lithium chloride, each at 0.025 M. The chloride was used to build up the ionic strength, since the diethylbarbituric acid concentration is fixed by its relatively low solubility at 0°C.

phoretic determination was made under conditions identical with those described above. The electrophoretic pattern obtained in this experiment is illustrated in Fig. 2.

It is immediately obvious from Figs. 1 and 2, that the results obtained in the two experiments were strikingly different. As a result of heating, one component (III) had completely disappeared and another component (II) had been



increased in amount; on the other hand, components I and IV were unaffected by heating under these conditions. A quantitative estimation of the relative amounts of components II and III in these two preparations was obtained from the measurement of the areas in the electrophoretic patterns. This indicated that the increase in component II which occurred on heating was approximately equal to the loss of component III.

When the observations described above were first made we were not certain whether L- and S-activities were associated with one or with two substances.

Therefore, we were inclined to believe from these data that component III corresponded to L-antigen and that component II corresponded to S-antigen; furthermore, that as a result of heating, L had been transformed to S. Let it be said at once that this hypothesis was subsequently proved wrong. Nevertheless, we immediately attempted to isolate by electrophoretic separation the four components shown in Fig. 1, in order to identify the substances serologically. These results may be briefly summarized as follows: components I, the fastest, and IV, the slowest, were readily isolated and were found to be serologically inert. Sharp electrophoretic separation of components II and III was not possible, due to the fact that their respective mobilities were not sufficiently different for such resolution. Even when electrophoresis was carried out in solutions with pH values from 6.0 to 8.6, such fractions which were obtained invariably showed L- and S-activity in equal amounts. Since the above methods were inadequate for the purpose, fractionation of the L- and S-antigens from crude dermal filtrate was attempted by other means.

Fractionation of Dermal Filtrate by Precipitation.—It has been shown by Craigie and Wishart (1) that the L- and S-antigens can be concentrated from crude dermal filtrate by precipitating at pH 4.5 and redissolving the precipitate at pH 6.5. This procedure enabled these workers to eliminate a considerable amount of serologically inert nitrogenous material, present in the crude filtrate, without appreciably reducing the titer of the antigens in the final preparation. Parker and Rivers (3), in their experiments dealing with the purification of S-antigen from heated dermal filtrate, had also observed that the heat-stable active substance has similar solubility characteristics. Procedures based on these observations were employed in the fractionation of concentrated dermal filtrate and the fractions thus obtained were studied electrophoretically and serologically. The following experiment illustrates the methods employed and the results obtained.

Experiment 3.—1150 cc. of vaccine dermal filtrate, collected over a period of 5 weeks, were concentrated by evaporation from cellophane bags to a volume of 110 cc. The slight amount of insoluble material present in the concentrate was removed by centrifugation and discarded. The clear concentrate was again placed in a cellophane sack and dialyzed overnight against 4 liters of unbuffered physiological saline solution at 3°C. The sack and its contents were then suspended in 450 cc. of 0.05 M acetate buffer solution at pH 4.63, also at 3°C. Precipitation began at once at the surface of the cellophane membrane. The sack was agitated from time to time and by the end of 3 hours no further increase in the precipitate could be noted. The material was then removed from the sack and the precipitate was separated by centrifugation in the cold. The clear, straw-colored supernatant fluid, which had a pH of approximately 4.6, was set aside for further study; this was designated fraction A. The oyster-gray sediment, which occupied a volume of about 2 cc. in the centrifuge tube, was washed in the cold with 45 cc. of the same acetate buffer solution. The resedi-

mented, washed material was then taken up in 50 cc. of 0.05 M cacodylate buffer solution at pH 6.31 and dialyzed overnight against 450 cc. of the same solution at 3°C. Approximately half of the material dissolved under these conditions. The material insoluble at pH 6.31 and the substance dissolved at this pH were separated by centrifugation. The clear, pale straw-colored supernatant solution was labeled fraction B and stored. The sediment was washed with the cacodylate buffer solution and then suspended in 50 cc. 0.05 M veronal buffer solution of pH 8.56. Following dialysis against a large volume of the same buffer solution an appreciable part, but not all, of the material dissolved. The clear liquid was again separated from the precipitate by centrifugation and designated fraction C. The precipitate which was insoluble at pH 8.56 dissolved partially in 0.1 M borate buffer solution pH 11.0 and almost completely in 0.05 M NaOH; since all the material in dermal filtrate was originally soluble in neutral solutions, this portion probably consisted largely of denatured products.

The three fractions which had been saved for further study were: A, the material which remained soluble at pH 4.63; B, the material which was insoluble at pH 4.63 but soluble at pH 6.31; and C, the material which was insoluble at pH 4.63 and 6.31, but soluble at pH 8.56. Portions of fractions A and B were placed in cellophane sacks and dialyzed against 0.05 M lithium-veronal buffer solution at pH 7.91 for 1 or 2 days in the cold, after which they were examined by electrophoresis; similar data were obtained on fraction C in veronal buffer solution pH 8.56. The serological properties of all three fractions were investigated.

Fraction A was found to contain only two components; these had the same electrophoretic mobilities as components I and IV in whole dermal filtrate. Fraction B consisted of a single substance which was electrically homogeneous and which moved at a rate corresponding to that of component III. Fraction C contained a trace of component III, but most of the material appeared to correspond to component II in whole filtrate. The electrophoretic patterns obtained with fractions A, B, and C are shown in Figs. 3, 4, and 5, respectively.

The results of serological studies on the original material and on fractions A, B, and C are summarized in Table I. Briefly it may be stated that fraction B, consisting of the electrically homogeneous component III, contained practically all of the L- and S-reacting material present in the original concentrated dermal filtrate. It gave a precipitate when diluted 1:1600 and incubated in the presence of an optimum amount of L- or of S-antibody. The small amount of L- and S-precipitinogen activity found in the C fraction was completely lost after refractionation; furthermore, all of the serologically active material was now recovered in the solution corresponding to the original B fraction. The A fraction was serologically inert.²

This portion of the work may be summarized as follows. Components I and

² In several early experiments in which dialysis of crude filtrate against pH 4.6 buffer solution was inadequate appreciable amounts of components II and III were left in fraction A.

IV found in vaccine dermal filtrate may be separated quantitatively from components II and III by precipitation of the latter at pH 4.6. Furthermore, component III can be isolated from this precipitate in uncontaminated form by extracting this precipitate at pH 6.3. Such a solution of component III contains practically all of the L- and S-reactive substance present in the original filtrate.

Observations Indicating that a Single Substance in Its Native State Contains Both L- and S-Activity.—In our laboratory, precipitation titrations with the

TABLE I
Serological Activity of Fractions Derived from Vaccine Dermal Filtrate

Test solution	Anti-serum	Dilution of test solution							
		1:25	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200
Concentrated dermal filtrate	L		++++	++++	++++	++++	+++	++	+
	S		±	+++	++++	++++	++++	++++	+
Fraction A	L	—	—	—	—	—			
	S	—	—	—	—	—			
Fraction B	L		++++	++++	++++	++++	+++	++	±
	S		±	++++	++++	++++	++++	++	±
Fraction C	L	++++	++++	+++	+	±			
	S	++++	++++	+++	++	+			
Refract-ionated C	L	±	—	—	—	—			
	S	+	±	—	—	—			

L-antiserum 1601 was used in a dilution of 1:2 throughout. S-antiserum 493 was used in a dilution of 1:8. 0.01 M lithium-veronal buffer solution, pH 8.6, containing 0.9 per cent of NaCl served as diluent. Test mixtures were incubated at 50°C. overnight.

See text for preparation of test materials.

soluble antigens of vaccinia are ordinarily carried out by incubating the antigen-antibody mixtures at 50°C. for 18 hours in closed tubes. Such an incubation period is sufficient to degrade the heat-labile antigen, L, to a state where it no longer precipitates with its antibody. Consequently, at this stage in the experiments it was necessary to consider the possibility that the material designated as component III was a substance which possessed only L-activity in its native form. According to such an hypothesis, this native material could be degraded by heating to a substance with the electrical mobility of component II and with S-activity.

In order to test this hypothesis a solution containing component III was diluted serially in the usual manner, and portions of each dilution were placed in tubes with

optimal amounts of L- or of S-antibody. Four sets of duplicate titrations were thus made and each set was incubated for 18 hours at a different temperature, namely, at 3°, 20°, 37°, and 50°C.

In each set of titrations the precipitin endpoint in the presence of S-antibody was identical with that obtained in the presence of L-antibody. Flocculation was slower at the lower temperatures, and with incubation at 3°C. periods of 48 to 72 hours were necessary in order to develop maximum precipitation; end-points thus obtained equalled those observed after 18 hours at 50°C.

The presence of an S-reactive substance in solutions of component III which were incubated at temperatures that do not change the precipitability of the heat-labile substance, clearly shows that component III in its native state possesses both L- and S-reacting portions; therefore, S cannot be a degradation product of L-antigen. Observations reported in succeeding sections will clarify the relationship between component II and heated component III.

Evidence based on absorption experiments in which solutions of component III were used with L- and with S-antisera indicates that the two antigenic parts of component III cannot be separated by this method. The following protocol illustrates the results obtained with one of several preparations of antigen absorbed with antisera.

Experiment 4.—40 cc. of an electrophoretically homogeneous solution of component III were obtained from 800 cc. of vaccine dermal filtrate by the method described in the protocol of Experiment 3. The solution had a titer of 1:2048 with L- and with S-antibody. A 0.7 cc. volume of S-antiserum 493 was mixed with 0.1 cc. of the solution of antigen in one instance, and in another with 0.05 cc. of antigen and 0.05 cc. of saline. Each antigen-antibody mixture was diluted with an equal volume of saline solution, and incubated at 37°C. for 4 hours, and then at room temperature for a similar period of time. The voluminous precipitate which formed was removed by centrifugation. Similar absorptions were carried out with 0.8 cc. volumes of L-antiserum 1601 and varying amounts (0.01 to 0.10 cc.) of solution of antigen, sufficient saline solution being added to each mixture to bring the final volume to 1.6 cc. These were tested for residual antigen and for residual antibody. The results of these tests which are summarized in Table II show that absorption with either L- or S-antibody removes both L- and S-antigenic substances completely or reduces both to the same degree.

It is apparent from these observations that the electrically homogeneous³ component III, which was obtained from the dermal filtrate, contains both L- and S-activity and that the two serologically distinct parts are inseparable in the native substance. Ultracentrifugal and chemical studies on this material, which will be reported in another paper, showed it to be a homogeneous protein

³ Studies on the material at values of pH between 6.2^{*} and 8.6 consistently revealed but one electrophoretic component. These measurements are discussed in detail in a subsequent paper.

TABLE II
Absorption of Solutions of Component III with L- and S-Antibody

Absorption mixtures		Residual antigen										Residual antibody			
Antigen	Antiserum type	Anti-sera	Dilution of original antigen solution									Dilution of absorbed mixture			
α .	α .		1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096	1:1	1:2	1:4	1:8
	Unabsorbed	L	++++	++++	++++	++++	++++	++++	++++	+	-				
		S	-	-	\pm	++++	++++	++++	++++	+	-				
0.01	Anti S 0.7	L	-	-	-	-	-	-	-	-	-	-	-	-	-
		S	-	-	-	-	-	-	-	-	-	-	-	-	-
0.05	Anti S 0.7	L	-	-	-	-	-	-	-	-	-	++++	++++	-	-
		S	-	-	-	-	-	-	-	-	-	+	+	-	-
0.1	Anti L 0.8	L	++++	++++	++++	++++	+	\pm	\pm	-	-	-	-	-	-
		S	++++	++++	++++	++++	++	\pm	\pm	-	-	-	-	-	-
0.05	Anti L 0.8	L	++	++	\pm	-	-	-	-	-	-	-	-	-	-
		S	++	++	\pm	-	-	-	-	-	-	-	-	-	-
0.025	Anti L 0.8	L	-	-	-	-	-	-	-	-	-	++	-	-	-
		S	-	-	-	-	-	-	-	-	-	++	-	-	-

L-antiserum 1601 was prepared from pooled sera of rabbits hyperimmunized with active elementary bodies of vaccinia by absorption of S-antibody with heated dermal filtrate. S-antiserum 493 was obtained from a rabbit immunized with a non-infectious fraction of heated dermal filtrate rich in the heat-stable soluble antigen of vaccinia. Tests for residual antigen were carried out with optimal dilutions of antisera, i.e., 1:2 for L- and 1:8 for S-antisera. Determinations of residual antibody were made by adding 0.25 cc. of a 1:16 dilution of unheated vaccine dermal filtrate to equal volumes of serial dilutions of the absorbed mixture.

See text for details of absorption technique.

with a molecular weight near that of serum globulins. On the basis of electrophoresis and ultracentrifugal evidence, therefore, we conclude that component III is a single molecular substance containing both L- and S-activity. Henceforth we shall designate it as LS-antigen. This LS-antigen is probably the substance that Craigie and Wishart (1) prepared from the same type of source material and called "LS fraction." Our method of isolation of the antigen is similar to theirs and the serological data obtained following incubation at various temperatures and by absorption with L- and S-antibody agree with their findings. The present experiments supplement the observations of these authors in that they were done with solutions of antigen which were proved to be pure by a number of different criteria and hence these experiments carry additional weight in theoretical discussions of the nature of LS-antigen.

Effect of Heat on LS-Antigen.—Destruction of the precipitability of the labile soluble substance of vaccinia by heating has been well established (1, 5). The combination between heated L- antigen and L-antibody without subsequent flocculation has been discussed in the preceding paper (2). Since pure LS-antigen was now available it seemed desirable to study the effect of heat on its physical and serological characteristics.

Experiment 5.—The observations described in the following experiment are typical of those noted in the study of several different preparations of LS-antigen. A portion of the solution of LS-antigen from Experiment 4 was heated for $\frac{1}{2}$ hour in a water bath at 70°C. in a closed tube. The solution which had been clear now showed a faint opacity. After removing the trace of insoluble material by high speed centrifugation (12,000 R.P.M.) the solution was redialyzed against 0.05 M lithium-veronal buffer solution at pH 7.91 and was examined by electrophoresis.

The electrophoretic mobility of this lot of unheated LS-antigen under these conditions was 4.0×10^{-6} cm./sec. per volt/cm. After heating this solution, a single electrically homogeneous component was still present, but it now moved at a rate of 5.9×10^{-6} cm./sec. per volt/cm. This preparation of heated LS was inhomogeneous when examined in the analytical centrifuge, as will be shown in another paper in this series. The precipitin titer of the solution of unheated LS was 1:2048 with both L- and S-antibody; the heated LS still titered 1:2048 with S-antibody but failed to precipitate over the range of dilutions from 1:8 to 1:4096 in the presence of L-antibody.

This experiment throws considerable light on the early observations which misled us into believing that components II and III represented S- and L-antigens respectively. Heating destroyed the precipitability of the L-part of the LS-antigen and increased the mobility of the degraded molecule to a value almost identical with that of component II in crude dermal filtrate. The augmentation of component II concurrent with the disappearance of component III in heated crude filtrate thus appears to have been a coincidence.

The Occasional Occurrence of Partially Degraded LS-Antigen in Crude Dermal Filtrate.—Although pure LS-antigen had been previously obtained in four successive experiments by means of the fractionation technique which has been described, it was found in one experiment that the fraction which usually contained only LS-antigen was contaminated with a component having the mobility of either component II or of heated LS-antigen.

Experiment 6.—850 cc. of dermal filtrate were concentrated and fractionated in the manner described in Experiment 3. Fraction B was examined by electrophoresis in 0.05 M lithium-veronal buffer pH 7.89 and found to contain two components; one component corresponding to LS-antigen was present in an appreciable amount, and the other, corresponding to either heated LS-antigen or component II, was estimated to be present in about $\frac{1}{2}$ the concentration of LS-antigen. Although the titer of the solution was 1:1600 with L-antibody and 1:3200 with S-antibody this was considered inconsequential at the time. Accordingly, the solution was refractionated to remove the contaminating material. In short, the material was precipitated three times at pH 4.6 and each time the precipitate redissolved almost completely at pH 6.6. The final preparation gave essentially the same electrophoretic pattern and serological titers as did the original fraction B.

Refractionation of the solution which might have been expected to eliminate the contaminating material if it were component II failed to accomplish this. Furthermore, since the contaminant had solubility characteristics similar to those of LS-antigen it appeared likely that it was a degraded form of LS-antigen. Therefore, at this point, it seemed desirable to know whether the technique of fractionation, as regularly employed, was adequate to separate heat-degraded LS-antigen from native LS-antigen.

Experiment 6 (Continued).—14 cc. of the three times fractionated solution B from Experiment 6 were still available. The solution had an S-titer of 1:1600 and L-titer of 1:800. One half of the material was heated at 70°C. for $\frac{1}{2}$ hour. This portion then failed to react with L-antibody but still retained its S-titer of 1:1600. The two solutions were recombined; the precipitin titers were then 1:400 for L-antigen and 1:1600 for S-antigen, as might have been expected.

After dialysis, the mixture was again studied electrophoretically; the pattern revealed the presence of two components with mobilities corresponding to LS-antigen and heated LS-antigen, which were present in concentrations closely corresponding to their respective titers. The material was then subjected to the solubility fractionation process and was again examined electrophoretically. No change in the resulting pattern was noted. The serological titers remained the same.

Essentially similar electrophoretic and serological observations were made on a mixture containing equal amounts of solutions of unheated and heated LS-antigen from Experiment 3. In this case, however, the two electrical components were about equal quantitatively, and the solution had titers of 1:800 to 1:1600 with L- and S-antibody respectively.

The results obtained in Experiment 6 indicate that heat-degraded LS-antigen has essentially the same solubility characteristic as native LS-antigen and hence cannot be separated from it by the procedures employed. This is of importance for it indicates that, in general, dermal filtrate contains no partially degraded LS-substance, *i.e.*, one possessing S-activity but not L-activity. In other words, all of the L- and S-activity in fresh dermal filtrate is carried by component III. In the single experiment in which pure LS-antigen was not isolated by the process of fractionation it appears that the contaminant was a degraded form of antigen similar to heated LS-antigen. The reason for the appearance of this contaminant in a single pool of dermal filtrate is not at hand. It may be pointed out, however, that, unlike the usual preparations, in this case, several of the lots of the angle supernatant fluid from the suspension of crude pulp had not been filtered immediately, but were allowed to stand at ice box temperature for several days before being passed through a Seitz pad. The centrifuged but unfiltered fluid is slightly opalescent and contains some virus, tissue debris and bacteria, and the presence of one or more of these might well have contributed to partial denaturation of LS-antigen by enzymatic digestion or otherwise.

Inhibition of L- and S-Antibodies by Degraded Forms of LS-Antigen.—The interpretation of the experiments described in the previous paper (those dealing with inhibition of anti-soluble-substance antibodies of vaccinia by various preparations of materials from vaccine dermal filtrate) was difficult, due to the lack of critical evidence showing that the degradation products of a single substance were capable of inhibiting the two antibodies. Accordingly, solutions of pure LS-antigen were treated with heat alone, and also with heat and dilute alkali, and the specific inhibitory properties of the resulting preparations were studied (Experiments 7 and 8).

Experiment 7.—A portion of solution obtained from Experiment 3 was heated at 70°C. for $\frac{1}{2}$ hour. The resulting solution still precipitated in a dilution of 1:1600 with optimal amounts of S-antibody but failed to flocculate when dilutions of 1:25 to 1:3200 of the solution were mixed with optimal amounts of L-antibody. Inhibition tests were made in the following manner. Varying amounts of two different L-antisera were treated with varying amounts of heated LS-antigen solution. Sufficient saline solution was added to the test mixtures to make the final dilutions of serum comparable. The mixtures were incubated at 50°C. for $\frac{1}{2}$ hour and then at 37°C. for an additional $\frac{1}{2}$ hour. The solutions remained clear and centrifugation failed to sediment an appreciable amount of material. The test sera, together with controls, prepared by properly diluting antisera with saline and incubating for a similar period, were then set up in constant amounts with serial twofold dilutions of unheated crude dermal filtrate. Illustrative data obtained are summarized in Table III.

It is evident from the results summarized in Table III that a solution of LS-antigen, shown to be pure by electrophoresis and ultracentrifugation, when

degraded by heat to the stage where it fails to give a precipitin reaction with L-antibody, is still capable of combining with L-antibody as demonstrated by the inhibition technique. An additional point of interest may be mentioned regarding the serological behavior of LS-antigen degraded by heat. Mixtures of serial dilutions of such antigen and optimal amounts of L-antibody, which give no precipitate after incubation, were subsequently treated with optimal amounts of S-antibody and incubated in the usual manner. The precipitin titers in these instances were approximately the same as those obtained in control titrations in which no L-antibody was employed. Apparently, the presence of L-antibody, which was presumably combined with the degraded

TABLE III
Inhibition of L- and S-Antibody by Degraded Forms of LS-Antigen

Inhibiting solution	Ratio of inhibiting solution to antiserum	Anti-serum	Dilution of dermal filtrate					Saline + test mixture
			1:8	1:16	1:32	1:64	1:128	
None		L	++++	++++	++++	+++	++	
		S	++++	++++	++++	+++	++	
LS heated	1:1	L	+	—	—	—	—	—
	2:1	L	—	—	—	—	—	—
LS heated with alkali	1:1	L	++	++++	+++	++	++	—
	2:1	L	±	+++	+++	++	++	—
	1:1	S	—	—	—	—	—	—
	1/2:1	S	—	—	—	—	—	—
	1/4:1	S	±	+++	+	—	—	—
	1/8:1	S	++	++++	++++	+++	—	—

L-antiserum 1601 and S-antiserum 493 were used in the experiments summarized in this table.

See legend of Table II and text for details of experiment.

L-portion of the antigen, did not interfere with the union of the S-portion with its antibody and subsequent flocculation of the entire aggregate.

Experiment 8.—A portion of the solution of heated LS-antigen used in Experiment 7 was freed of buffer by dialysis and treated with a sufficient amount of sodium hydroxide to bring the final concentration to 0.1 M. The material was heated in a water bath at 50°C. in a closed vessel for 90 minutes with frequent gentle agitation. After neutralization to pH 7 the solution was titered with S-antibody. The treatment failed to effect a complete loss of specific precipitability of the solution, for the S-titer, although much diminished, was still 1:320. Accordingly, the procedure was repeated except that 0.05 M concentration of alkali was used. Although the first treatment did not result in any change in the appearance of the solution, this second treatment was accompanied by the production of a small amount of insoluble material which, incidentally, was arranged in small, elongated ribbon-like fibers which were white in

reflected light and colorless in transmitted light. The insoluble material was removed and the solution tested in dilutions of 1:5 to 1:640 at pH 7 with optimal amounts of S-antibody. No precipitation occurred.

An inhibition experiment of the type described in the previous section was now performed. L- and S-antisera were treated with varying amounts of alkali-heat degraded antigen and subsequently tested for precipitable antibody. The results of the experiment are summarized in Table III.

It is apparent from the data presented in Table III that as little as 0.1 cc. of the solution of LS-antigen which had been degraded by heat and alkali (approximately 0.15 mg. of dried material) was sufficient to inhibit completely the S-antibody in the mixture. Furthermore, even $\frac{1}{4}$ of this amount of antigen was sufficient to inhibit the antiserum slightly. On the other hand, 1.0 cc. (1.5 mg.) was incapable of inhibiting the amount of L-antibody employed. Thus, degradation of LS-antigen by means of heat and alkali completely destroys the serological activity of the L-portion of the molecule and changes the S-portion of the molecule to a form which no longer precipitates with S-antibody but is capable of inhibiting it. It will be noted that the inhibitory action of the degraded S-portion of the molecule on S-antibody is of a greater order of magnitude than that of the degraded L-portion for its corresponding antibody.

Electrophoretic studies on the solution of LS-antigen in Experiment 8 which had been degraded by heat and alkali showed that it contained one electrically homogeneous component with a mobility of 6.4×10^{-5} cm./sec. per volt/cm. at pH 7.9 and ionic strength 0.05. The mobility of this substance is close to that of component I in dermal filtrate. The two are not identical, however, for the latter failed to inhibit S-antibody. Furthermore, the two substances have different solubilities; the S-inhibitor is precipitated at pH 4.5 and fails to dissolve appreciably at pH 6.3, but it can be brought into solution between pH 8 and 9, and will then remain in solution when the pH is lowered to nearly 7.0.

DISCUSSION

The experiments presented in this paper indicate that all of the serological activity associated with the heat-stable and heat-labile soluble antigens of vaccinia are present in a single protein molecule. This differs somewhat from the concept of Craigie and Wishart (5), who considered the two antigens to occur, ordinarily, in the form of a complex which could be dissociated into two separate antigens under certain circumstances.

It is possible to state with assurance that various levels of degradation of LS-antigen can be accomplished, leaving either the L- or S-part of the molecule in a stage where it can still combine with the corresponding antibody without precipitation. The observations on inhibition of L- and S-antibody reported in the previous paper were not sufficiently conclusive to enable us to decide as to the nature and origin of the inhibiting substances. On the basis of the

present studies it is clear that they arise directly from the LS-antigen through partial degradation of the molecule.

Here it has been shown that the L-portion of the LS-molecule can be degraded by heat without serological alteration of the S-portion. In a subsequent paper in this series, it will be demonstrated that by means of enzymatic digestion the S-portion of the molecule can be degraded without altering the serological activity of the L-portion. On the basis of this latter observation it is possible to explain directly (2) the results occasionally obtained by other workers (4, 5), which had been interpreted (5) to indicate a dissociation of their LS-complex antigen into two separate components.

SUMMARY

Virus-free filtrate, obtained from suspensions of vaccine virus-infected dermal pulp of rabbits and rich in the soluble substances of vaccinia, was shown to contain four distinct components in electrophoresis experiments. Electrophoretic and serological observations served as a guide in developing a method for separating these components from one another. This method depended upon changes in the solubilities of the components with alterations of pH.

Three of the four components appeared to be serologically inert when tested with anti-vaccinia sera. All of the L- and S-activity was found to be associated with a single component which was electrically homogeneous at several values of pH and which was homogeneous in the ultracentrifuge.

This single substance, designated as LS-antigen, precipitates in equal titers with optimal amounts of L- and of S-antibody and is completely removed from solution by absorption with either antibody.

The LS-antigen of vaccinia appears to be a protein molecule with two antigenically distinct parts, L and S. Heating modifies the L-portion in such a manner that the substance no longer precipitates with L-antibody; this degraded antigen still combines with L-antibody, as is shown by inhibition tests, and still precipitates with S-antibody. Similarly, treatment with heat and dilute alkali modifies the S-portion of LS-antigen so that it combines but does not precipitate with S-antibody; and at the same time all recognizable immunological properties of the L-portion are destroyed.

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THE NEPHROTIC CRISIS

By KENDALL EMERSON, JR., M.D., AND DONALD D. VAN SLYKE, Ph.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

A typical complication of the nephrotic syndrome is the occurrence of acute febrile episodes with symptoms of peritonitis. The attack may take place without warning and with dramatic suddenness, ushered in by an abrupt rise in temperature to as high as 106°F. in 3 or 4 hours, often accompanied by a chill, and always by generalized abdominal pain and rigidity, nausea, vomiting and prostration. To this typical picture Farr (10) has given the name "nephrotic crisis."

There is a marked leucocytosis ranging from 20,000 to 40,000. In many instances, but not in all, blood cultures, especially if taken early, and cultures of peritoneal fluid will demonstrate the presence of a pathogenic organism; it is most commonly a pneumococcus, but not infrequently other organisms are found. The hemolytic streptococcus, bacillus coli, hemophilus influenzae and bacillus Morgagni have all been obtained in cases in this hospital. In some instances the acute illness lasts 36 to 48 hours, and is followed by a recovery almost as abrupt as the onset. In others there is a persistence of high fever and bacteremia with death occurring in the course of 10 days to 2 weeks. Such generalized infections have been seen in nephrotic patients ranging in age from 2 to 26 years and appear to be as common among males as females. Recurrences in the same patient are frequent and may or may not be accompanied by infection with the same organism.

Some patients show all the above signs and symptoms of peritonitis without demonstrable organisms in the blood or peritoneum. Such patients as a rule recover in 12 to 24 hours.

In this paper all such acute febrile episodes of the type described, whether or not infection was demonstrated, will be termed nephrotic crises.

A systematic study of the metabolic changes accompanying these acute episodes was carried out in this hospital by Farr (1) and by Farr and MacFadyen (2). These investigators observed that an accelerated loss of urinary non-protein nitrogen, resulting frequently in a negative nitrogen balance, regularly preceded by several days the onset of the crisis. They also noted that with onset of the crisis there was a further drop in the already low plasma albumin concentration to the neighborhood of 1 per cent or lower, and that a rise to the previous level accompanied recovery.

These findings indicated a disturbance in nitrogen metabolism before and during the nephrotic crisis, and led Farr and MacFadyen to an investigation of the non-protein nitrogenous constituents of the blood of nephrotic patients. Among the substances investigated the only significant changes found were in the plasma amino acids.

For the plasma amino acid analyses advantage was taken of the recently developed ninhydrin-carbon dioxide method of Van Slyke and Dillon (4) for the quantitative estimation of free alpha-amino acids. This method determines no amines other than the amino acids, and is therefore more specific than the nitrous acid procedure (3). MacFadyen and Van Slyke (5) devised an application of the ninhydrin-carbon-dioxide method to plasma, which was sufficiently rapid and simple to serve as a routine clinical test. The normal level of alpha-amino acid nitrogen in human plasma was found to range from 3.5 to 5.0 mg. per 100 cc. These normal figures have been confirmed by Dr. Goettsch (6) at the Babies Hospital in New York City, using the same method.

By means of this technique, Farr and MacFadyen (2) found that in patients with the nephrotic syndrome, during periods without crises, the plasma amino acid nitrogen level was consistently below normal, ranging from 2.5 to 3.5 mg. per 100 cc. They also found that during the acute nephrotic crisis, with or without demonstrable infection, there occurred a further drop to a figure usually below 2.5 mg. per 100 cc. The figure 2.5 they arbitrarily termed the "critical level." Recovery was found to be associated with an abrupt rise in plasma amino acids in the direction of normal. These changes in plasma amino acids constituted the most characteristic metabolic abnormality noted in the nephrotic crisis, whether or not infection was demonstrated. With few exceptions, in cases in which plasma amino acid values significantly below the critical level have been observed, typical crises either have been present or have occurred within the next 2 or 3 days. Conversely, in observations of 44 crises, none has been seen in which the plasma amino acid level failed to drop, or which did not terminate with recovery when the amino acid level rose above the critical value. The accompanying charts illustrate these points graphically.

Figure 1 shows the febrile response to a series of five consecutive crises, accompanied by transient bacteriemia, in a 3 year old nephrotic girl observed by one of the writers (E) over the course of 3 months. Each episode was a typical nephrotic crisis, and during the periods of temperature elevation all the signs and symptoms of peritonitis were present, whereas during the periods of normal temperature the child appeared perfectly well. The remarkably close correlation between the elevation of temperature and depression of amino acids below the critical level is apparent.

Figure 2 illustrates the same phenomenon in another patient, a 4 year old boy, with a somewhat more prolonged period of infection.

Figure 3 illustrates a nephrotic crisis which is unusual in several respects. The patient was in an older age group. Her symptoms began as

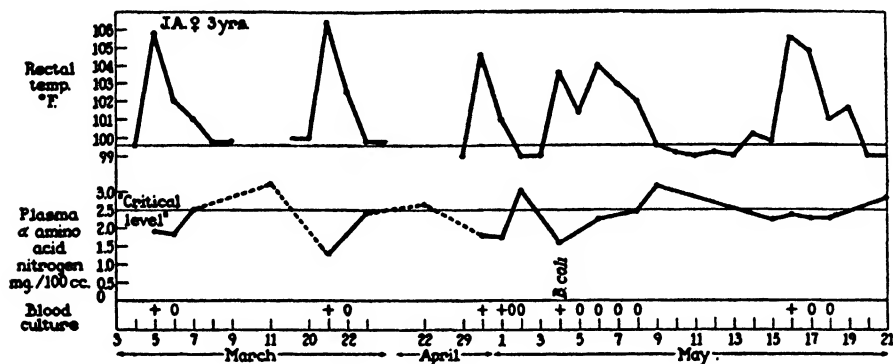


FIG. 1

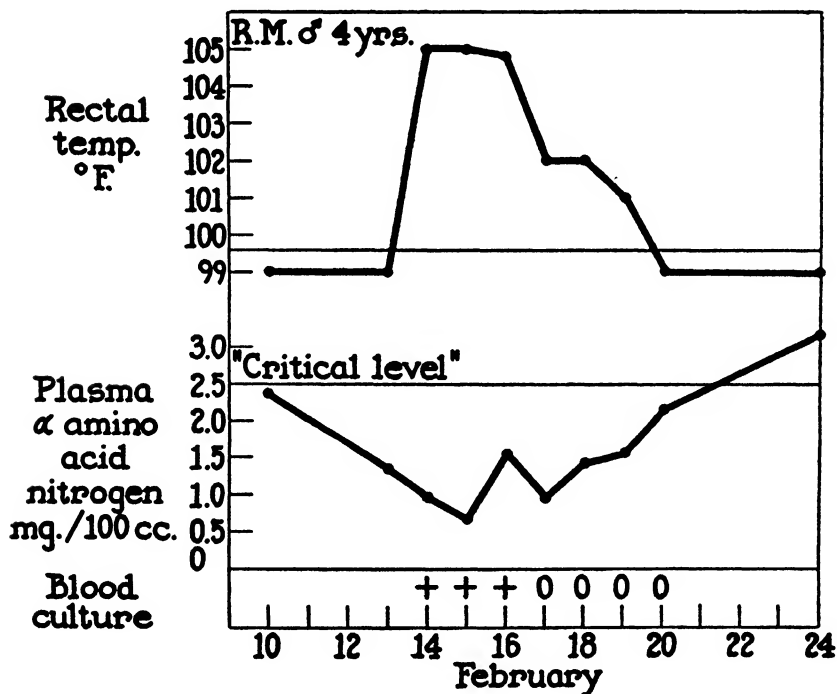


FIG. 2

a pelvic peritonitis associated with menstruation and remained localized for 2 days. During this time the plasma amino acid level, although showing a downward trend, continued slightly above the critical level. Sub-

sequently there was a further fall and the level remained depressed until death occurred on the ninth day of the crisis. This crisis is the only one which has terminated fatally in this hospital since the plasma amino acid changes have been under observation, accompanied by amino acid therapy.

The so-called critical level of 2.5 mg. of amino acid nitrogen per 100 cc. of plasma is a figure established by observations on children exclusively

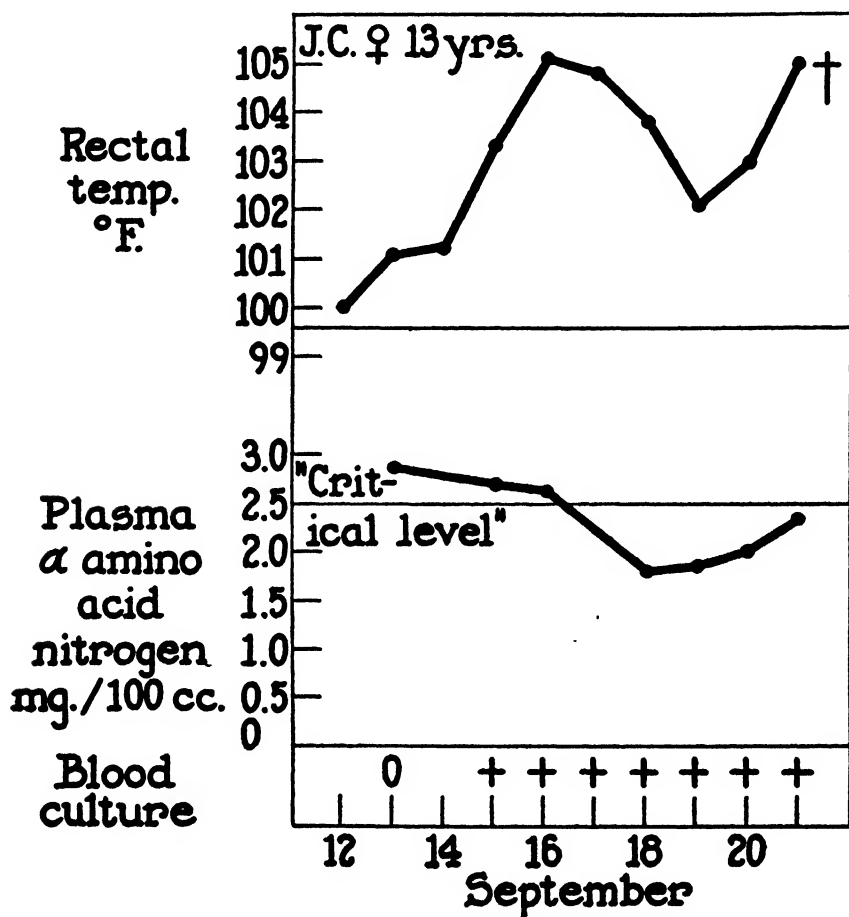


FIG. 3

under 10 years of age. The critical level may not be found at 2.5 mg. in all children when a larger number has been observed, and it may be different in adults. For this reason it may be more important, at least as far as prognosis in a given crisis is concerned, to emphasize the trend of the plasma amino acid curve rather than the absolute level at any one moment.

The demonstration of a plasma amino acid deficiency in the nephrotic syndrome has led logically to attempts to replace the missing amino acids. Gastrointestinal symptoms are so acute that feeding is impracticable; consequently the amino acids have been given intravenously. Elman (7) in St. Louis was the first to show the clinical practicability of administering amino acids intravenously in the form of a casein hydrolysate. Farr, Emerson, and Fitcher (8) utilized this material,¹ both in attempts to raise the plasma amino acid level by prolonged treatment in patients not in crisis, and in the treatment of the acute nephrotic crisis. In patients not in crisis no effect occurred on the level of plasma amino acids or proteins or on the apparent clinical course of the disease. Nevertheless, it was shown that the nitrogen supplied by this method was utilized as efficiently and completely as dietary nitrogen (8, 9), so that it presumably served to combat the malnutrition that is so typical of nephrosis. In the treatment of the nephrotic crisis, when adequate food ingestion is usually impossible, nitrogen catabolism is accelerated, and malnutrition tends to be exacerbated, the use of intravenous amino acids seems logical if for no other reason than to maintain nutrition. It cannot be claimed that this form of treatment has either prevented the occurrence or shortened the course of the nephrotic crisis. However, the fact remains that prior to the introduction of amino acid therapy the mortality from crises with acute infection was 66 per cent in this hospital (10), whereas in 32 crises among 11 patients under 10 years of age treated with amino acids and with no other change in therapy there have been no deaths. The older patient depicted in figure 3 has been the only fatality among cases treated with amino acids. Besides belonging in an older age group, she had an infection which was resistant to sulfapyridine.

DISCUSSION

MacLeod and Farr (11) observed that nephrotic children carry for long preliminary periods in their throats the same organisms that ultimately invade their blood streams during nephrotic crises. When a crisis is accompanied by bacteriemia, the latter appears to result not from a fresh, virulent infection, but rather from a sudden decrease in resistance to an organism towards which the patient had previously acted as a carrier. Hence it appears possible that the bacterial invasion may result from a breakdown of immunity caused either by the hypoaminoacidemia or by the unknown metabolic disturbance which may cause both the forerunning negative nitrogen balance and the hypoaminoacidemia.

In some instances (e.g., fig. 2) where measurements were made from 1 to 3 days prior to the onset of a crisis, the plasma amino acids had already begun to fall. This would indicate that this drop is a reflection of

¹ The casein hydrolysate, used in these studies, was kindly supplied by Mead Johnson & Co., Evansville, Ill.

an underlying metabolic disturbance which precedes the clinical symptoms of the nephrotic crisis.

In the course of the ordinary upper respiratory or other infections in nephrotic children no changes in the plasma amino acid level have been noted (2). However, preliminary studies have given some indication that amino acid metabolism may be affected in conditions other than nephrosis, for it has been found (12) that during the acute phase of pneumonia the plasma amino acid level is depressed, and that with recovery there is a return to normal levels.

In 19 out of 44 nephrotic crises observed in 14 patients no infection could be demonstrated by repeated blood cultures. In such cases, one cannot exclude the possibility of a transient bacteriemia which may have disappeared before the first blood culture was taken; the observed bacteriemia is sometimes so transient that, after a positive culture at onset, a second culture 6 hours later may be negative. However, in view of 43 per cent of crises with negative cultures, the possibility cannot be ignored that the whole clinical picture in cases without demonstrable bacteriemia may be capable of production by a purely metabolic disturbance, without bacterial invasion as either a primary or secondary factor.

SUMMARY

The acute febrile attack with severe gastro-intestinal symptoms is described which occurs and recurs typically in nephrotic children, with or without demonstrable bacteriemia. Work by Farr, MacFadyen, MacLeod and their collaborators is cited showing that the attack is preceded by a metabolic disturbance evidenced by a loss of body nitrogen and by a dramatic fall in the plasma amino acid content. Recovery is regularly accompanied by a rise in the plasma amino acid content. Intravenous nutrition with amino acids seemed beneficial in reducing the mortality.

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MICRODETERMINATION OF CALCIUM BY PRECIPITATION AS PICROLONATE AND ESTIMATION OF THE PRECIPITATED CARBON BY MANOMETRIC COMBUSTION*

By DONALD D. VAN SLYKE AND FRANK J. KREYSA

(From the Hospital of The Rockefeller Institute for Medical Research)

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The rapid and accurate gasometric procedure for microdetermination of organic carbon developed by Van Slyke, Page, and Kirk (13) and recently improved by Van Slyke and Folch (11) opens the way to an indefinite number of quantitative analyses in which substances are precipitated in centrifuge-combustion tubes with organic precipitants, and the amounts of the precipitates are measured by determinations of their carbon. This procedure is especially adaptable to microanalyses, because the combustion is accurate for samples containing amounts of carbon down to 0.1 mg., and precipitants can be used in which the organic component contains many carbon atoms for 1 atom of the element determined. Kirk (8) and Hoagland (5) have used this principle for micro phosphorus determinations in which strychnine phosphomolybdate, with 28.4 (empirical factor) times as much carbon as phosphorus, is precipitated and burned, permitting analyses of samples with less than 0.01 mg. of phosphorus. Similarly Hoagland has used combustion of benzidine sulfate for microdeterminations of sulfate (5) and of magnesium hydroxyquinolate for magnesium (6).

In the present method calcium is precipitated from aqueous solution as the picrolonate, $\text{Ca}(\text{C}_{10}\text{H}_7\text{O}_5\text{N}_4)_2 \cdot 8\text{H}_2\text{O}$, and the amount

* This paper was submitted by Frank J. Kreysa in partial fulfillment of the requirements for the degree of Master of Science in the department of Professor Joseph B. Niederl, Graduate School of Arts and Science, New York University.

of the precipitate is estimated from the CO_2 yielded by the combustion method of Van Slyke and Folch (11). The picrolonate is precipitated and burned without transfer in a Pyrex centrifuge tube. The fact that the picrolonate contains 20 carbon atoms to 1 calcium makes the method applicable to samples with amounts of calcium down to 0.02 mg.

Several authors have used picrolonate for quantitative determination of calcium. Dworzak and Reich-Rohrwig (4) weighed it as a precipitate. Alten, Weiland, and Knippenberg (1) developed a colorimetric method to measure the red color formed when the picrolonate was treated with bromine water. Bolliger (3) used known amounts of lithium picrolonate to precipitate the calcium, and estimated the picrolonate left in solution to calculate the amount precipitated by difference.

The properties of calcium picrolonate have been studied by Robinson and Scott (10) and by Dworzak and Reich-Rohrwig (4). There is some uncertainty as to whether there are 7 or 8 molecules of water of crystallization, but the water content is of no significance when the precipitate is measured by carbon analysis. The solubility in water at room temperature is reported by Robinson and Scott (10) to correspond to 5 mg. of calcium per liter. However, our results indicate that the presence of free picrolonic acid in solution depresses the solubility of the calcium picrolonate to a small fraction of this value.

To avoid resolution of any of the precipitate by wash water, washing is replaced by a complete drainage of the supernatant fluid. The picrolonic acid in the precipitated solution is so dilute (0.005 N), and the amount of it which remains adherent to the minute precipitate and to the clean walls of the tube is so slight and constant, that the carbon in it can be included without significant error in the correction for reagents obtained by blank analyses.

Apparatus

1. For the gasometric determination of carbon in the precipitate the Van Slyke-Neill (12) manometric apparatus is used, with the accessories for combustion described by Van Slyke and Folch (11). The shape of the combustion tube, however, is altered by tapering its bottom into a cone as shown in Fig. 1, so that it can serve also

as a centrifuge tube. The bottom of the tube must be of the dimensions and shape indicated in Fig. 1, in order to hold the precipitates and to permit complete decantation of the supernatant fluid. The form of combustion-centrifuge tube shown in Fig. 1 is somewhat more convenient than that used by Hoagland (5). Complete decantation is easier, and the combustion can be carried out with a free flame, as in Van Slyke and Folch's description of the combustion (11), without need of the protecting device between the flame and tube which Hoagland found necessary.

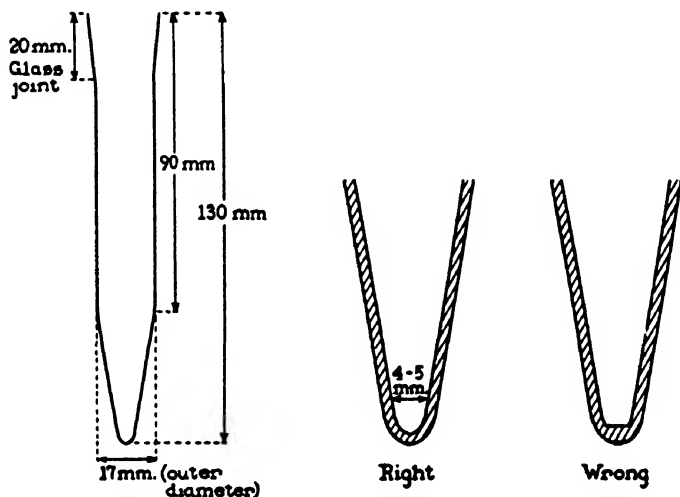


FIG. 1. Combustion-centrifuge tube

2. A drying oven with a temperature of 100–105°.
3. For use when the calcium-containing material contains organic matter and must be ashed, an electric muffle in which the temperature can be set at 500°.
4. Pyrex glass stirring rods of about 2 mm. diameter to mix the precipitating solutions in the combustion-centrifuge tubes. It is well to make a small button on one end of each rod, and always use the other end for the stirring, in order to avoid contaminating the solutions with organic matter from the fingers.

The combustion-centrifuge tubes and the rods are cleaned with hot chromic acid mixture as described by Van Slyke and Folch ((11) p. 533). However, the desiccators with calcium chloride used by Van Slyke and Folch cannot be used when the tubes

are to be used for calcium determinations. The calcium chloride can be replaced by phosphorus pentoxide, or the washed tubes can simply be inverted in a covered beaker or wide mouthed Erlenmeyer flask and dried in an oven.

Reagents

1. *Approximately 0.008 N picrolonic acid.* The purity of the picrolonic acid used is important, in order to obtain properly crystalline calcium picrolonate precipitates. Some impure preparations of picrolonic acid have been tried which regularly gave amorphous precipitates of calcium picrolonate, impossible to handle in the centrifuge tubes by the technique employed. A preparation of Kahlbaum's picrolonic acid "for analysis" could be used without purification. Other preparations had to be recrystallized.

To prepare the solution 2.1 gm. of picrolonic acid and 1 liter of water are placed in an Erlenmeyer flask covered with a watch-glass to retard evaporation, and are heated for 2 or 3 hours on a steam bath. The flask is rotated occasionally to assist solution. The solution is cooled to room temperature and filtered through a calcium-free filter paper to remove any undissolved residue. The solution is kept in an ice box, so that any material which is insoluble at 0° will precipitate before the solution is used.

2. *Approximately 0.04 N hydrochloric acid.*

3. *Concentrated sulfuric acid in a dropping bottle.*

4. *Reagents for the Van Slyke-Folch combustion (11).*

Procedure

A sample of solution containing 20 to 120 γ of calcium is measured into the combustion-centrifuge tube and concentrated to dryness by placing the tube in the oven at 100–105°. If any organic matter is present, it is then destroyed by adding a drop of concentrated sulfuric acid and ashing at 480–520° in the electric oven.

When serum is analyzed, 0.2 or 0.5 cc. is measured into the centrifuge-combustion tube and 1 drop of concentrated sulfuric acid is added. The tube is put into the oven at 100–105° for 2 hours, and is then transferred to the electric furnace, where the temperature is raised slowly to 480–520°. It is left overnight at this temperature, which accomplishes the ashing with-

out softening the Pyrex glass. If particles of carbon still remain in the ash, the tube is returned to the furnace until the ash is white, but the initial 12 to 14 hours usually suffice. The added sulfuric acid accelerates the destruction of organic matter and also serves to protect the glass against the etching which might result if an alkaline ash or one high in chlorides were formed.

To dissolve the ash 1 cc. of 0.04 N hydrochloric acid is added. One of the cleaned stirring rods is used to loosen up the ash, and is left in the tube while the latter is immersed in boiling water for 10 minutes. Then 1 cc. of hot distilled water is added and the heating is continued for another 10 minutes to complete solution of the ash.

The solution is cooled to room temperature and 2 cc. of the ice-cold filtered solution of picrolonic acid are added. The liquids are stirred occasionally with the rod until crystals of calcium picrolonate appear, which occurs in 3 to 5 minutes.

After a half hour at room temperature the tube is cooled to near 0° by immersion in ice water, and is kept at this temperature for an hour and a half. During the first half hour it is stirred two or three times. The stirring rod is then withdrawn and rinsed into the tube with 1 cc. of the 0.008 N picrolonic acid solution. The tube is capped and left for an hour longer at 0° to complete the crystallization.

The tube is then centrifuged for 15 minutes at 3000 R.P.M. and the supernatant fluid is removed by suction through a fine curved capillary. To avoid loss of any of the crystals floating on the surface film, suction is not started until the capillary is immersed well below the surface. Then as the fluid is withdrawn the particles in the surface film stick to the wall of the centrifuge tube and are not lost. Suction is stopped when 0.2 to 0.3 cc. of fluid remains over the precipitate.

To drain off the rest of the supernatant solution the tube is inverted to an angle of about 30° with the horizontal and the lip is rested on a wet towel or filter paper. If a drop of liquid adheres in the narrow bottom of the tube, it is touched with a curved platinum wire, which breaks the surface film and starts drainage. After 2 minutes at the 30° angle the tube is shifted to a nearly vertical position and left there for 15 minutes to complete the drainage. The mouth of the inverted tube is sprayed with a few

drops of water to remove adherent picrolonic acid solution from the lip. Drainage in this manner leaves in the tube a uniform film of approximately 0.025 cc. of liquid. This film contains 0.031 mg. of picrolonic acid, equivalent to 2.3 γ of calcium. Variations in the film are not great enough to cause variations of more than ± 1 mm. in the blank, equivalent to 0.2 γ of calcium.

After the tube has drained, the carbon in the precipitate is determined by combustion as described by Van Slyke and Folch (11), the centrifuge tube serving as combustion tube. The pressure readings are made with the gases at 2 cc. volume.

As a routine we have dried the tubes by immersion in a steam bath before the combustion, but this is not necessary, as the slight film of aqueous solution does not contain enough water to interfere with the efficiency of the combustion fluid.

Blank Analysis—3 cc. of the 0.008 M picrolonic acid and 2 cc. of water are placed in a centrifuge-combustion tube which has been cleaned by use in a previous combustion or by heating in chromic-sulfuric acid. The picrolonic acid solution is decanted and the tube is drained exactly as in the analyses, and is subjected to combustion. The value of $p_1 - p_2$, measured with gas volume at 2 cc., obtained in this blank combustion is the c correction. It includes a correction of about 10 mm. for the carbon in the picrolonic acid of the film left in the tube after draining.

Calculation

The micrograms of calcium in the sample are calculated by multiplying the CO_2 pressure, P_{CO_2} , from the burned precipitate by a factor given in Table I.¹

$$\text{Micrograms Ca} = P_{\text{CO}_2} \times \text{factor}$$

$$P_{\text{CO}_2} = (p_1 - p_2 - c)$$

p_1 and p_2 are the manometer readings before and after absorption of the carbon dioxide and c is the value of $p_1 - p_2$ obtained in the blank analysis.

¹ The factors for micrograms of calcium given in Table I are the factors for mg. of carbon of Van Slyke and Folch ((11) p. 529) multiplied by 1000 \times 0.16686,

$$0.16686 \quad \frac{40.08}{240.20} = \frac{\text{weight of 1 gm. atom calcium}}{\text{weight of 20 gm. atoms carbon}}$$

TABLE I
Factors for Calcium Calculation

Temperature	Factor; micrograms Ca indicated by 1 mm. PCO_2 , when $a = 2.000$ cc.*
°C.	
10	0.2460
11	46
12	33
13	21
14	09
15	0.2398
16	86
17	76
18	64
19	53
20	41
21	31
22	19
23	09
24	0.2299
25	89
26	79
27	69
28	59
29	51
30	41
31	31
32	23
33	14
34	04
35	0.2196

* If a is not exactly 2 cc., multiply the factor in the table by $a/2$.

EXPERIMENTAL

I. Determination of Calcium in Standard Calcium Solutions with from 20 to 100 γ of Calcium

A stock solution containing approximately 1 mg. of Ca per cc. was prepared as follows: Selected crystals of Iceland spar were washed in dilute hydrochloric acid (about 0.1 N), then with water,

and were dried over phosphorus pentoxide. An accurately weighed sample of about 2.5 gm. of these crystals was transferred to a 1 liter volumetric flask, dissolved in 15 cc. of concentrated hydrochloric acid, and diluted with water to 1 liter.

From this stock solution, 1, 2, 3, 4, and 5 cc. were accurately pipetted into 100 cc. volumetric flasks and diluted to the mark with water to make the dilute standards containing 10 to 50 γ of Ca per cc.

For the analyses in Table II, 2 cc. portions of the standard solutions were pipetted into the centrifuge-combustion tubes followed by 2 cc. of ice-cold 0.008 N picrolonic acid solution, and the procedure of analysis was followed from that point as described above for routine analyses.

The results are summarized in Table II.

II. Serum Analyses: (1) Comparison of Picrolonic Acid Method with Results Obtained by Microtitration of Calcium Oxalate by Potassium Permanganate; (2) Recovery of Added Calcium

1. Calcium determinations on blood serum were performed as described in this paper and the results obtained are summarized in Table III.

For the microtitration with permanganate 2 cc. samples of serum were analyzed as described in Peters and Van Slyke (9), with washing of the oxalate by Clarke and Collip's decantation method.

2. Results from recovery of calcium added are summarized in Table IV.

Estimation of Calcium in Low Calcium Tap Water by Combustion of Calcium Picrolonate

According to Dworzak and Reich-Rohrwig (4) calcium can be determined gravimetrically as picrolonate in ordinary drinking and tap water. The silicic acid and iron to be expected do not interfere. These authors determined calcium in Vienna's mineral waters, treating them directly with picrolonic acid without any preliminary operations.

For control determinations by titration, 100 cc. portions of the water were concentrated to dryness on the steam bath and the residue was redissolved in 1 cc. of 1 N hydrochloric acid. The

TABLE II
Determination of Calcium in Standard Solutions

Calcium content of sample	P_{CO_2}	a^*	Temperature	Factor from Table I	Calcium	
					Found	Error
γ	mm.	cc.	$^{\circ}C.$		γ	per cent
100.8	444.7	2.006	28.6	0.2261	100.5	-0.3
100.8	442.6	2.006	28.8	59	100.0	-0.8
80.1	360.6	2.000	32.0	23	80.2	+0.1
80.1	359.0	2.000	32.3	20	79.8	-0.4
60.1	268.3	2.000	29.6	45	60.2	+0.2
60.1	267.6	2.000	29.5	46	60.1	± 0.0
40.1	179.7	2.000	29.2	49	40.4	+0.8
40.1	177.6	2.000	28.9	52	40.0	-0.3
20.0	88.5	2.000	31.8	24	19.7	-1.5
20.0	90.9	2.000	32.0	23	20.2	+1.0

* Exact volume at which CO_2 pressures were measured.

TABLE III
Comparison of Calcium Determination in 2 Cc. Samples of Serum by Oxalate Titration Method with Determinations in Samples of 0.5 and 0.2 Cc. by Present Method

Strain No.	Calcium content by present method						Calcium by titration, per 100 cc. serum
	Volume of sample	P_{CO_2} (duplicates)	a	Temperature	Ca in sample	Ca in 100 cc. serum (average of duplicates)	
	cc.	mm.	cc.	$^{\circ}C.$	γ	mg.	mg
1*	0.5	123.9	2.000	26.0	28.3		
	0.5	126.6	2.000	26.2	28.8	5.72	5.61
2	0.5	220.6	2.000	29.0	49.7		
	0.5	218.2	2.000	29.0	49.1	9.88	9.94
3	0.5	226.8	2.006	31.3	50.7		
	0.5	225.8	2.006	31.4	50.5	10.12	10.16
4	0.5	235.0	2.006	27.4	53.4		
	0.5	232.0	2.006	27.5	52.7	10.62	10.58
5	0.5	242.6	2.006	30.4	54.5		
	0.5	243.8	2.006	30.5	54.7	10.92	11.04
6*	0.2	60.2	2.006	31.4	13.5		
	0.2	61.8	2.006	31.4	13.8	6.85	6.75
7	0.2	100.0	2.006	30.6	22.4		
	0.2	97.9	2.006	30.9	21.9	11.1	11.04
8	0.2	88.7	2.006	30.9	19.8		
	0.2	90.4	2.006	31.1	20.2	10.0	10.16

* Nephritic with low serum calcium.

solution was washed into a centrifuge tube and brought to pH approximately 5 by adding sodium acetate. The calcium was precipitated as oxalate which was washed three times by centrifugation with saturated calcium oxalate solution, and titrated as described by Peters and Van Slyke (9). Triplicate determina-

TABLE IV
Recovery of Calcium Added to Serum

Serum No.	Calcium present in 0.5 cc. serum before addition	Calcium added per 0.5 cc. serum	Calcium found after addition, per 0.5 cc. serum	Recovery of added Ca	
				Per 0.5 cc. serum, average of duplicates	Per cent of added Ca
	γ	γ	γ	γ	
1	50.6	50.4	101.5 101.9	51.1	101.3
2	52.5	50.4	104.2 104.5	51.7	102.5
3*	28.5	40.0	68.1 67.9	39.5	98.8
4	54.6	50.4	103.9 103.8	49.3	97.8

* Nephritic with abnormally low serum Ca.

TABLE V
Determination of Calcium in New York City Tap Water

Calcium determined in 100 cc. samples by permanganate titration = 12.45 mg. per liter.

Volume of sample	PCO_2	α	Temperature	Factor	Ca in sample	Ca per liter
cc.	mm.	cc.	$^{\circ}C.$		γ	mg.
2.0	134.5	2.006	27.8	0.2268	25.0	12.52
2.0	134.5	2.006	27.9	67	25.0	12.52

tions indicated 12.42, 12.41, and 12.51, average 12.45, mg. of Ca per liter of water.

For microanalyses by picrolonate combustion 2 cc. portions of New York City tap water were treated in the same way as the calcium standard solutions. The results given in Table V serve as an example of a water of relatively low calcium content.

Remarks

The 0.008 *M* picrolonic acid solution used as precipitant is approximately saturated at 0°. For the exact solubility of pure picrolonic acid in water Hugouneng, Florence, and Couture (7) gave 1.2 gm. per liter at 17°, but other authors (1, 4) have used 0.01 *M* solutions (2.64 gm. per liter) as analytical reagents.

If the concentration of free HCl were too great in the precipitating solution, free picrolonic acid would be precipitated with the calcium picrolonate. We have found that HCl up to 0.02 *N* concentration does not precipitate picrolonic acid under the conditions of the present analysis, but that 0.05 *N* HCl would cause error from precipitation of free picrolonic acid. The conditions for redissolving the ash of serum are designed to keep the HCl concentration within 0.02 *N* at the time of precipitation.

Under the conditions used for serum analysis there is no interference from the amounts of magnesium present.

The chief difficulty encountered in making the method practical was in ascertaining the conditions to insure a dense crystalline precipitate, suitable for centrifuging and decanting. Pure picrolonic acid was found to be essential; one commercial preparation gave only amorphous precipitates until it was purified. Furthermore, the concentrations of the calcium and the picrolonic acid and the temperature of the solution influence the character of the precipitate. The prescribed stirring with a glass rod is necessary to obtain uniformly the crystalline form of the precipitate.

In ashing serum, temperatures up to 520° could be used without softening the Pyrex glass, and repeated carbon determinations on the acid ash gave negative results. Temperatures much higher than 520° soften the glass enough to spoil the fit of the ground glass joint of the tube. Baernstein and Grands (2), in a paper which has appeared since this work was completed, use a similar ashing technique, but with silica tubes and a temperature of 600°, which gave complete ashing in 3 hours. If many calcium determinations were to be run routinely, the time saved by substituting silica for Pyrex would probably be worth the extra cost.

SUMMARY

A micromethod for calcium is described in which the calcium is precipitated as picrolonate and the precipitate, containing 20 atoms of carbon to 1 of calcium, is estimated from the carbon, which is determined by the rapid manometric wet combustion method of Van Slyke and Folch. Precipitation and combustion are done without transfer in a single centrifuge-combustion tube.

The method serves for estimation of smaller amounts of calcium than can be determined accurately by the usual microprocedures based on titration of the oxalate; 0.2 mg. of calcium or 0.2 cc. of serum suffices for an analysis.

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THE DISSOCIATION CONSTANTS OF HYDROXYLYSINE

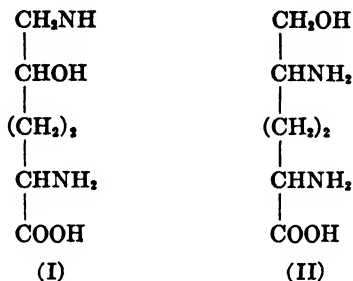
By FRIEDRICH W. KLEMPERER, A. BAIRD HASTINGS, AND
DONALD D. VAN SLYKE

(From the Department of Biological Chemistry, Harvard Medical School, Boston,
and the Hospital of The Rockefeller Institute for Medical Research, New York)

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The position of the hydroxy group in the amino acid hydroxylysine, whose isolation was reported in 1938 (1), has not been definitely established.

The chemical evidence points to one of two positions as indicated by Formulas I and II.



It is the purpose of the present communication to present values of the dissociation constants of the acidic and basic groups of hydroxylysine, and to compare these values with the corresponding constants of lysine. The influence on the acid-base dissociation constants of the introduction of the hydroxy group into the lysine molecule provides additional evidence for the correctness of Formula I or II.

Material

The hydroxylysine used was a preparation of the crystallized monochloride prepared from gelatin by Van Slyke, Hiller, Dillon, and MacFadyen (1). On analysis, the yields were as follows:

C ₆ H ₁₁ O ₃ N ₁ ·HCl.	Calculated.	C 36.27, H 7.61, N 14.11, NH ₂ -N 14.11, Cl 17.85
Found.	“ 36.29, “ 7.72, “ 14.12, “ 13.95, “ 18.23	

For the carbon, hydrogen, and nitrogen values, by Pregl micro combustion, we thank Dr. A. Elek. The amino nitrogen was determined by the manometric nitrous acid method (2). The chloride content was by the microtitration of Sendroy (3).

Methods

The dissociation constants were calculated from the titration curves of lysine and hydroxylysine.

The nature and scarcity of the material used made certain adaptations of the conventional technique necessary. Since rather strongly acid and alkaline dissociation constants had to be determined, dilution of the amino acid could not be carried below a molarity of 0.01 without a sacrifice of accuracy. Therefore, a small titration vessel (Fig. 1) was constructed from a 25×120 mm. centrifuge tube, to which were fused inlet and outlet tubes for hydrogen. The rubber stopper of the vessel carried two platinized platinum electrodes (a) (or in some experiments also a bulb type glass

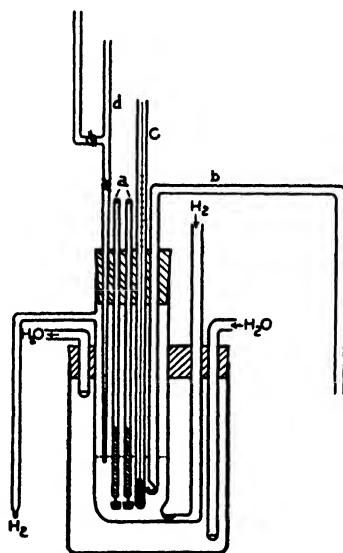


FIG. 1. Microtitration vessel

electrode), a KCl agar bridge (b) connecting to a saturated calomel cell, a thermometer (c), and an automatic 1 cc. micro burette (d), the tip of which was finely drawn out and immersed in the solution. The titration vessel was surrounded by a jacket through which water at 38° was circulated. This assembly was kept in an electrically shielded air thermostat at 38° . The potentials of the hydrogen electrodes were measured with a Leeds and Northrup type K potentiometer and type R galvanometer. When pH values were measured with the glass electrode, potentials were taken with an additional vacuum tube amplifier which was included in the potentiometric circuit.

3 to 4 mg. of hydroxylysine monohydrochloride were weighed out on the micro balance and dissolved in 10 cc. of 0.1 N KCl. After equilibration

with hydrogen, the solution was titrated successively with 0.1 N HCl and 0.1 N NaOH. Titrations of lysine dihydrochloride were performed with 0.1 N NaOH, followed by back titration with acid.

All pH measurements were referred to standard acetate buffer to which was assigned a value of pH 4.65 according to Hitchcock and Taylor (4).

The results were calculated according to Van Slyke's equation (5)

$$K = \frac{[H^+] \times ([B] + [H^+] - [OH^-])}{C - ([B] + [H] - [OH^-])}$$

TABLE I

Titration of Hydroxylysine Monohydrochloride

C = the total concentration of ampholyte; B = the concentration of NaOH. A negative value of B designates the concentration of HCl.

pH	C	B	pK'_1	pK'_2	pK'_3
	<i>mm per l.</i>	<i>mm per l.</i>			
2.34	1.80	-0.76	2.18		
2.49	1.77	-0.54	2.12		
2.59	1.76	-0.44	2.11		
2.72	1.75	-0.33	2.12		
2.90	1.73	-0.22	2.25		
3.08	1.72	-0.15	2.17		
8.42	1.69	0.75		8.63	
8.55	1.68	0.90		8.65	
8.74	1.68	1.14		8.67	
8.86	1.68	1.30		8.65	9.65
9.03	1.67	1.54		8.64	9.66
9.19	1.67	1.78		8.64	9.67
9.35	1.67	2.02		8.66	9.64
9.56	1.66	2.34		8.65	9.66
9.72	1.66	2.58			9.69
9.88	1.65	2.81			9.70
10.03	1.65	3.12			9.66

where K = the dissociation constant, $[B]$ = the concentration of NaOH added; it assumes a negative value when HCl was added to the solution. C = the total concentration of ampholyte. The value of $[H^+]$ was calculated from $\alpha_H \times 0.84$ where $\alpha_H = 10^{-pH}$ and 0.84 was the activity coefficient of $[H^+]$ under the conditions of our experiment. Similarly, the value of $[OH^-]$ was calculated from $\alpha_{OH} \times 0.81$ where $\alpha_{OH} = 10^{13.55-pH}$ and 0.81 was the activity coefficient of $[OH^-]$. The value of 13.55 for pK_w was determined experimentally by measuring pH values of various concentrations of NaOH in 0.1 N KCl under conditions identical with those of the experiment. From these data, it was calculated according to the equation, $pK_w = pH - \log [NaOH] - \log 0.81$.

The dissociation constants of the two amino groups, the buffer actions of which overlap, were determined by successive approximation according to the procedure of Hastings and Van Slyke (6).

Results

The data from a representative titration of hydroxylysine are given in Table I and Fig. 2. In the acid range, the glass electrode and hydrogen elec-

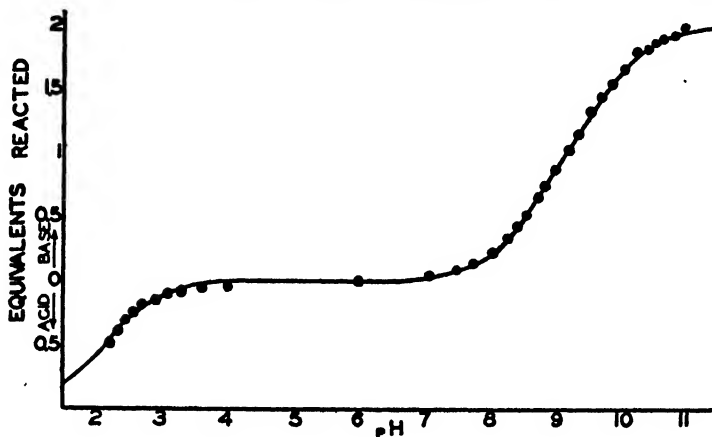


FIG. 2. Titration curve of hydroxylysine. The smooth curve is based on the constants, $pK'_1 = 2.13$, $pK'_2 = 8.62$, and $pK'_3 = 9.67$. The points are experimental.

TABLE II
Dissociation Constants of Hydroxylysine and Lysine

	pK'_1	pK'_2	pK'_3
Hydroxylysine	2.16	8.65	9.67
	2.22	8.63	9.66
	2.00	8.59	9.68
Average	2.13	8.62	9.67
Lysine	2.17	8.91	10.32
	2.23	8.90	10.24
Average	2.20	8.90	10.28

trode pH values agreed within 0.01 pH; beyond pH 8, the deviation between the two methods became considerable. Although seven complete titrations of hydroxylysine were made, the data of only those titrations carried out at 38° with the hydrogen electrode will be reported. The values of the three dissociation constants, pK_1 , pK_2 , pK_3 , corresponding to the carboxyl group and the first and second amino groups respectively, calculated from three experiments are given in Table II. The pK values of lysine, reported by

Schmidt, Kirk, and Appleman (7) as 2.18, 8.95, and 10.53 for 25° could not be used for comparison with our data on hydroxylysine because of the differences in temperature and ionic strength of the solutions. Lysine was, therefore, titrated under the conditions of our titrations of hydroxylysine. The results of these titrations are also given in Table II.

DISCUSSION

The introduction of an OH group into the lysine molecule does not significantly alter the pK' of the carboxylic group (pK'_1). It decreases the pK' of the α -amino group (pK'_2) by 0.28 unit. On the other hand, the dissociation constant of the second amino group of hydroxylysine is depressed from pK 10.28 to 9.67; *i.e.*, 0.61 unit. From this, it would seem that the OH group is attached more closely to the second amino group than to the α -amino group, making its attachment to the δ -carbon the most likely position. Unfortunately, very few data are available about the influence of OH groups in various positions on an aliphatic amino group. A comparison with the dissociation constants¹ of ethylamine ($pK' = 10.66$) and ethanolamine ($pK' = 9.48$) shows that an OH group adjacent to the amino group may depress its pK by as much as 1.18 units.

However, it is shown by the dissociation constant of alanine ($pK'_2 = 9.72$) and serine ($pK'_2 = 9.15$) that the change in pK' of an amino group brought about by an adjacent OH group is smaller in an amino acid than in an otherwise unsubstituted aliphatic amine.

The dissociation constants of hydroxylysine are, therefore, consistent with Formulas I and II. The dissociation constants do not permit a differentiation between these two structures.

SUMMARY

The dissociation constants of hydroxylysine were determined at 38° as $pK'_1 = 2.13$, $pK'_2 = 8.62$, and $pK'_3 = 9.67$ as compared with those of lysine, $pK'_1 = 2.20$, $pK'_2 = 8.90$, and $pK'_3 = 10.28$. These data confirm the view that the hydroxy group is attached in the δ or ϵ position to the carbon atom adjacent to that carrying the amino group.

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¹ These values were taken from the review of Cohn (8).

INOSITOL, A CONSTITUENT OF A BRAIN PHOSPHATIDE

BY JORDI FOLCH AND D. W. WOOLLEY

(From the Hospital and the Laboratories of The Rockefeller Institute for
Medical Research)

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By the use of a recently developed method¹ it was found that phosphatides both from brain and spinal cord contained inositol. Brain cephalin² was found to be especially rich in it. When brain cephalin was fractionated by precipitation from CHCl_3 solution by increasing concentrations of alcohol,³ followed by dialysis of the fractions, inositol was found only in the fraction least soluble in alcohol. Both phosphatidyl serine⁴ and the fraction most soluble in alcohol were practically free of it. This evidence pointed to the existence of a new phosphatide in which inositol was a constituent. Inositol-containing phosphatides have been reported in tubercle bacilli⁵ and in soy beans,⁶ but have not been found previously in animal tissues.

The following facts showed that inositol was chemically combined in the lipid: (1) By microbiological assay the intact lipid was only one-twentieth as active as its hydrolytic products; (2) added inositol could be removed by dialysis, leaving the inositol content of the phosphatide unchanged; and (3) reaction of HIO_4 ⁷ with the lipid showed no free inositol.

The phosphatide fraction obtained by the methods indicated above was a friable white powder containing 4.5 per cent P and about 1 per cent N, all of it amino N. Various preparations contained from 6.8 to 8.6 per cent inositol. It represented about one-fourth of brain cephalin, or 0.4 per cent of the net weight of the brain. By other methods preparations have been obtained which contained up to 10 per cent inositol.

¹ Woolley, D. W., *J. Biol. Chem.*, **140**, 453 (1941).

² Thudichum, J. L. W., A treatise on the chemical constitution of the brain, London (1884).

³ Folch, J., *Proc. Am. Soc. Biol. Chem.*, in press (1942).

⁴ Folch, J., *J. Biol. Chem.*, **139**, 973 (1941).

⁵ Anderson, R. J., *J. Am. Chem. Soc.*, **52**, 1607 (1930).

⁶ Klenk, E., and Sakai, R., *Z. physiol. Chem.*, **258**, 33 (1939).

⁷ Malaprade, L., *Bull. Soc. chim.*, **1**, 833 (1934).

Finally, inositol was isolated by the following procedure. An aqueous emulsion of 3.5 gm. of the fraction (containing 6.8 per cent inositol) was precipitated by adding HCl up to 3 N concentration and the washed precipitate was hydrolyzed for 48 hours with boiling 6 N HCl. The hydrolysate was filtered and the filtrate treated with Ag_2O and H_2S in succession and finally concentrated and treated with 6 volumes of alcohol. Crystals formed which were collected after 10 days and recrystallized once from alcoholic HCl and once from alcohol, and finally dried at 100° in a vacuum. The yield was 140 mg.; m.p. 217.6° (uncorrected); mixed m.p. with inositol, 217.6° (uncorrected). Found, C 39.98, H 6.80 (corrected for 2.1 per cent ash). The hexaacetate was prepared; m.p. $211\text{--}213^\circ$ (uncorrected); mixed m.p. with inositol hexaacetate, $211\text{--}213^\circ$ (uncorrected). By microbiological assay the crystals were 100 per cent *meso*inositol within the limit of error of the method.

A STUDY OF THE TOTAL VOLUME OF THE HUMAN FINGER TIP AND TOE TIP

By G. E. BURCH, M.D.,* A. E. COHN, M.D., AND C. NEUMANN, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

During the course of vascular studies on the human finger tip and toe tip certain statistical constants were noticed which were considered worth recording separately. The studies were limited to the right index finger tip and right second toe tip. The finger tip previously defined (Sodeman, '37; Turner, Burch and Sodeman, '37) consists of that portion of the finger distal to a plane passing through the major dorsal and palmar distal creases. The toe tip was arbitrarily defined by comparable skin markings, viz., that portion of the toe distal to a plane passing through the major dorsal and plantar distal creases. In both instances the plane passes proximal to the distal interphalangeal joint.

Since the terminal portion of the toe is larger than the more proximal portion, the method described for determining the volume of the finger tip by measuring the volume of a rigid negative case (Sodeman, '37) could not be employed in these observations. In ascertaining the volume of the finger tip and toe tip negative casts of the parts were first made of a resilient moulage, Negocoll,¹ from which positive casts were made of dental stone. The positives were cut through the creases previously described. The volume of these positive casts was measured by the displacement of mercury which was then weighted. All measurements were made with the subject resting in the supine position and with the part at the level of his heart.

The apparatus employed is shown in Fig. 1. It was constructed of clear Lucite. Part A is essentially a cylinder with a closed broad base. Part B is a bevel-shaped ring made to fit snugly inside of A. It is used to hold the cast in place under the surface of the mercury when container A is being filled. Part B is bevelled so as to prevent the formation, and facilitate the removal, of bubbles of air during the filling of A with mercury. In use, ring B is placed inside of container A, and A is filled with mercury. The meniscus of the mercury is levelled to the top of A by pressing a thick, broad, glass slide over the top of the container. The volume of mercury then contained in A is a fixed quantity and is used for the measurements. Some of the mercury contained in A is poured out and saved, ring B is removed, and the positive cast to be measured is placed in A. Ring B is replaced in A, over the cast, and A is re-

* Fellow of the Commonwealth Fund.

¹ A preparation of Kern Co., New York.

filled with part of the mercury that has been saved. During the refilling of A with mercury, care is taken to keep the cast away from the wall of the container and to remove air bubbles. The mercury meniscus is again levelled off to the top of A with a glass slide. The mercury that is left over represents the volume of the cast. The volume of the mercury is determined from its weight, corrections being made for room temperature. On application of the method to the determinations of the volume of small metal cylinders of known volumes (3 to 7 cc.) the maximum error was less than 3 per cent.

Volume measurements were made of the right index finger tip and second toe tip of 29 normal, white, human subjects ranging in age from 17 to 55 years. The individual variations in sex, age, and volume of the parts are re-

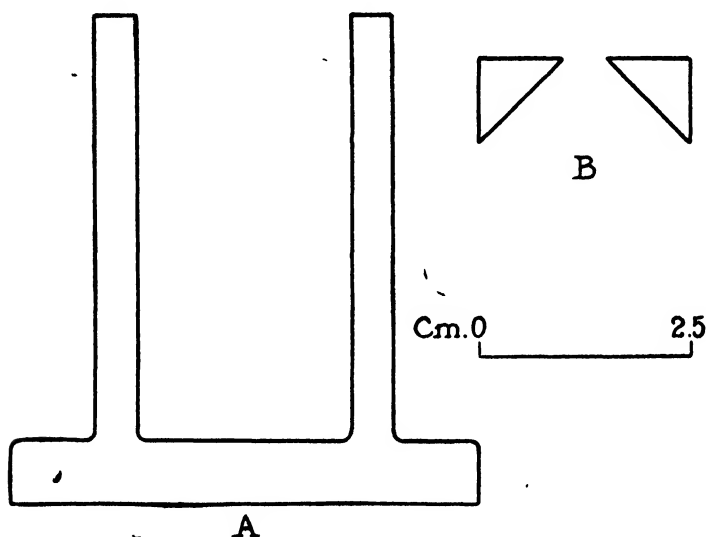


FIG. 1. Schematic representation of the apparatus (see text)

corded in Table 1. The mean volume of the finger tip for the entire group is 4366 ± 113 cu. mm. with a standard deviation of 911 ± 80 cu. mm. and a coefficient of variation of 20.60 ± 1.89 per cent. The mean volume of the toe tip for the group is 4059 ± 90 cu. mm. with a standard deviation of 720 ± 64 cu. mm. and a coefficient of variation of 17.75 ± 1.67 per cent. The statistical constants for the volume of the finger tip and toe tip for the entire group and for each sex group separately, are recorded in Table 2. The coefficient of correlation for the volume of the finger tip and volume of toe tip is $+0.760 \pm 0.053$ (Fig. 2).

The males and females were considered separately to learn whether there was a difference between the sexes in the volumes of the parts studied. The data for the 9 females (subject number 1 was eliminated because of her youth)

were studied statistically by a method for the study of small array of biological data (Mainland, '37). The mean volume of the finger tip is 3448 ± 99 cu. mm. for the females and 4868 ± 126 for the males, a difference of 1420 cu.

TABLE I
Total Volume of the Finger Tip and Toe Tip of 29 Normal White Human Subjects

Subject no.	Age (yrs.)	Sex	Volume of finger tip (cu. mm.)	Volume of toe tip (cu. mm.)
1	17	F	2821	2952
2	22	F	3108	3611
3	23	F	3222	3162
4	26	F	4174	3027
5	31	F	2922	2948
6	33	F	3789	4364
7	39	F	3530	4182
8	40	F	3080	3938
9	44	F	3222	2988
10	50	F	3981	3160
11	22	M	5364	5312
12	23	M	3781	3595
13	26	M	4846	3694
14	26	M	5516	5538
15	29	M	5242	4788
16	30	M	4163	4104
17	30	M	4600	4220
18	31	M	4640	4338
19	31	M	5090	4132
20	31	M	4895	4426
21	31	M	3624	3950
22	32	M	4503	4528
23	36	M	5034	4256
24	37	M	6022	4842
25	39	M	4113	4497
26	39	M	4020	4051
27	43	M	5034	4664
28	43	M	4412	3454
29	55	M	7088	5224

	<i>Volume of finger tip</i>	<i>Volume of toe tip</i>
Mean.....	4366 \pm 113	4059 \pm 90 cu. mm.
Range.....	2821 to 7088	2948 to 5538 cu. mm.
Standard deviation.....	911 \pm 80	720 \pm 64 cu. mm.
Coefficient of variation.....	20.60 \pm 1.89 per cent	17.75 \pm 1.67 per cent

mm. The probable error of this difference is ± 160 cu. mm., indicating a significant difference between volumes of the finger tips for the two sex groups. The mean volume of the toe tip is 3487 ± 124 cu. mm. for the females, and

4405 ± 88 for the males, a difference of 918 cu. mm. The probable error of this difference is ± 152 cu. mm., indicating a significant difference between the volumes of the toe tips for the two sexes.

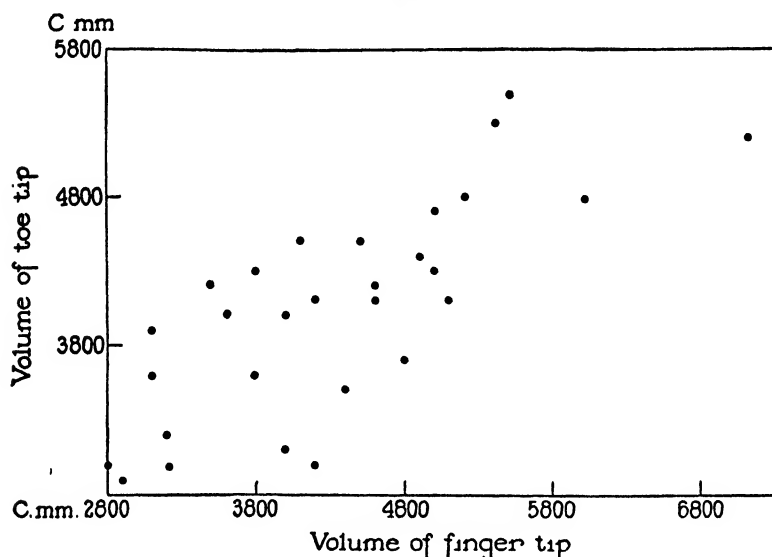


FIG. 2. Spot graph of the relationship of the volumes of the finger tips to the toe tips

TABLE 2

Statistical Constants for the Total Volume of the Finger Tip and Toe Tip for the Entire Group and Each Sex Separately

	Mean (cu. mm.)		Range (cu. mm.)		Standard deviation (cu. mm.)		Coefficient of variation (per cent)	
	Finger tip	Toe tip	Finger tip	Toe tip	Finger tip	Toe tip	Finger tip	Toe tip
Entire group.	4366 \pm 113	4059 \pm 90	2821 to 7088	2948 to 5538	911 \pm 80	720 \pm 64	20.60 \pm 1.89	17.75 \pm 1.67
Females.....	3488 \pm 99	3487 \pm 124	2922 to 4174	2948 to 4364	436 \pm 69	512 \pm 88	12.65 \pm 2.04	15.79 \pm 2.55
Males.....	4868 \pm 126	4405 \pm 88	3624 to 7088	3434 to 5538	803 \pm 88	566 \pm 62	16.49 \pm 1.85	12.84 \pm 1.45

COMMENT

The mean volume for the finger tip of this group of 29 normal individuals is 903 cu. mm. less than the mean volume previously reported for a different series of 26 normal, male individuals (Burch and Sodeman). This difference is accounted for in large part by the fact that this group contains 10 female subjects who have been shown to have smaller finger tips than the males. When we consider the mean volume for the finger tip of the males only in our series, this difference is reduced by 56 per cent.

The coefficient of variation for the toe tip is essentially the same as that for the finger tip. This is about equal to those for the weights of the heart, kidneys, liver, and body of normal individuals, which are 17.70, 17.80, 14.80 and 12.78 respectively (cited by Pearl).

The coefficient of correlation for the volumes of the finger tip and toe tip, $+0.760 \pm 0.053$, is greater than the coefficients of correlation for heart weight and body weight, heart weight and kidney weight, heart weight and liver weight, and heart weight and brain weight, which are all essentially expressions of volume. The four latter correlation coefficients are $+0.65$, $+0.56$, $+0.52$, and $+0.08$ respectively (Greenwood and Brown, '13). One might expect a high correlation between volumes of homologous finger tips and toe tips as they are more closely related anatomically and embryologically than heart and liver, heart and brain, and the like.

In all of these subjects there was some degree of distortion of the shape of the toes due to the wearing of shoes. The extent to which this has influenced the volume of the toe tips and in turn the statistical constants reported above is not known.

SUMMARY

A method is described for the measurement of the volume of the finger tip, toe tip, and other irregular portions of the body. The method consists of first making a negative cast of the part to be measured with a resilient moulage, then a positive cast of dental stone, and finally measuring the volume of the positive cast by displacing mercury. Volume determinations were made of the right index finger tip and right second toe tip of 29 normal, white subjects and statistical constants were calculated. The male group is found to possess significantly larger finger tips and toe tips than the female group. It is also found that the volumes of the finger tips and toe tips exhibit a high degree of correlation, $+0.760 \pm 0.053$.

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A STUDY OF THE RATE OF WATER LOSS FROM THE SURFACES OF THE FINGER TIPS AND TOE TIPS OF NORMAL AND SENILE SUBJECTS AND PATIENTS WITH ARTERIAL HYPERTENSION

By G. E. BURCH,* M.D., A. E. COHN, M.D., AND C. NEUMANN, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

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The role of the sympathetic nervous system in the pathogenesis of arterial hypertension has not been adequately defined. There is evidence to show that there may be an increase in sympathetic tone.^{1, 2} Most of these observations have been limited to the influence of the sympathetic nervous system on the cardiovascular system. The degree of sympathetic activity, especially in patients with arterial hypertension, may be estimated by measuring the rate of water loss from the skin. Such studies seem not to have been undertaken. It has been widely observed that resting and apparently comfortable patients with hypertension have cold and clammy extremities and that cutting certain sympathetic nerves leads to dryness of the extremities, but this has been observed likewise in other people. It was thought that quantitative measurements of the rate of sweating in normal and senile subjects and in patients with hypertension might throw light on the role of the sympathetic nervous system, and these investigations were therefore undertaken.

Method

The method used in these studies has already been described.³ It consists essentially of passing dry oxygen through chambers covering the skin of fingers and toes, and then conducting the moisture-laden oxygen through cold coils. From the difference in weight of the coils before and after the passage of oxygen, the amount of water lost is learned. First, the subjects rested for thirty minutes in a special room⁴ (temperature 75°F. $\pm 1^\circ$, relative humidity less than 50 per cent). This room was designed to remove all the appearances of a laboratory. The subjects were alone and undisturbed throughout the periods of observation; collections of sweat, weighing, and other manipulations were carried out in an adjoining room, separated from the subject's room by a sound-proof wall. The collections were made continuously for sixty minutes at fifteen-minute intervals. These observations were made during the last week of March, April, and the first two weeks of May, 1941.

This is the fourth paper reporting the results of studies of the small blood vessels and related subjects.

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* Commonwealth Fund Fellow.

There were thirteen normal subjects whose ages ranged from 8 to 44 years, ten patients with arterial hypertension who were free from renal or cardiac failure and were between 27 and 54 years of age, and eight senile subjects who were free from renal or cardiac failure and were normal except for the usual manifestations of senility. The ages of the senile subjects varied from 70 to 85 years. In addition, a miscellaneous group was studied. These observations included measurements on two subjects with hypertension which were made before, and three weeks after, bilateral sympathectomy (ninth thoracic sympathetic nerve to first lumbar, and the celiac and aortic renal plexuses), on a subject with advanced Raynaud's disease and scleroderma, and on a normal subject, continuously, for a period of 12.25 hours, and also simultaneous measurements on four groups of two subjects each, in separate beds in the same room.

The measurements were made simultaneously on the tip of the right index finger and right second toe of all subjects. In some instances it was the left index finger or left second toe (or both) that was examined simultaneously with the right ones. The areas of the finger and the toe nails were subtracted from the respective total areas of the parts examined, so that the results apply to the effective area only. The total areas were measured as previously described,³ and the areas of the nails, by measuring the area of paper necessary to cover the nails of the positive casts of the parts. The temperature of the oxygen surrounding the parts in the chambers was 75°F., and the relative humidity, approximately zero. The temperature of the air surrounding the adjacent parts was 75°F., and the relative humidity, 50 per cent. In order to ascertain the influence of the dry oxygen on the rate of water loss from the parts enclosed in the chambers, observations were conducted in which room air and dry oxygen were used alternately to collect the water given off by the enclosed parts. It was found that the rate of water loss was the same whether the relative humidity was approximately zero or near 50 per cent.

RESULTS

The mean rate of water loss from the tips of the index fingers of the normal subjects was 3.59 mg. per square centimeter per fifteen minutes; the minimum and maximum variations were 1.84 and 6.96, respectively. The mean rate from the toe tips was 1.95 mg.,* and the minimum and maximum variations were 0.67 and 6.81, respectively (Table I).

In the patients with arterial hypertension the loss from the finger tips was 3.63 mg., with minimum and maximum variations of 1.75 and 8.80, respectively, and, from the toe tips, 2.29 mg., with minimum and maximum variations of 0.62 and 6.12, respectively (Table II).

In the senile subjects the loss from the finger tips was 2.27 mg., with minimum and maximum variations of 1.71 and 2.87, respectively, and, from the toe tips, 1.08 mg., with minimum and maximum variations of 0.68 and 1.55, respectively (Table III, Fig. 1).

* The calculation is always given in milligrams per square centimeter per fifteen minutes.

TABLE I

Rate of Sweating (Mg./Sq. Cm. Skin Area/15 Min.) Measured Simultaneously on the Right Index Finger and Right Second Toe Tip of 13 Normal, White, Resting Adults

Subject no.	Date	Age (years)	Sex	Blood pressure (mm. Hg)	Area of skin (sq. cm.)	First 15 min.	Second 15 min.	Third 15 min.	Fourth 15 min.	Mean 15 min.	Rate of sweating as % of finger rate
Finger tips											
1	4/30/41	31	M	135/72	10.13	4.63	3.64	2.89	6.50	4.42	
2	3/28/41	41	M	120/70	11.22	2.48	3.23	2.91	2.78	2.85	
3	4/ 1/41	33	M	125/75	10.23	2.48	2.13	2.09	1.87	2.15	
6	5/ 3/41	40	F	110/70	8.64	2.56	2.31	4.23	4.21	3.35	
14	5/ 7/41	23	F	110/78	7.56	4.03	3.66	2.76	2.74	3.30	
24	5/ 1/41	29	F	110/80	7.56	2.39	4.72	5.22	6.42	4.69	
31	5/15/41	18	M	125/60	8.85	4.31	5.88	5.10	6.96	5.56	
49	5/13/41	26	F	110/65	8.66	5.57	4.50	3.46	2.75	4.07	
54	5/ 2/41	27	F	110/76	9.08	5.81	5.30	5.15	5.59	5.47	
66	5/ 5/41	37	M	115/75	10.76		3.04	2.36	2.10	2.50	
86	5/ 7/41	23	F	110/70	7.62	3.33	2.93	2.23	1.84	2.58	
92	5/ 1/41	44	M	118/78	10.26	2.45	2.63	2.36		2.48	
96	5/ 1/41	23	F	110/80	6.90	3.25	3.42	2.22	1.93	2.70	
Mean						3.61	3.65	3.31	3.81	3.59	
Maximum										6.96	
Minimum										1.84	
Toe tips											
1					8.10	2.00	1.55	2.03	1.70	1.85	42
2					8.22	1.92	1.72	1.38	1.12	1.54	54
3					9.05	1.98	1.81	1.72	1.78	1.82	85
6					8.60	1.24	1.18	1.64	1.51	1.40	42
14					7.98	1.56	1.04	1.13	1.17	1.23	56
24					8.22	1.13	1.60	2.12	3.07	1.98	42
31					9.66	2.93	3.74	2.84	2.81	3.08	55
49					8.44	2.71	2.30	1.67	1.34	2.00	49
54					8.29	6.81	4.92	4.38	3.84	4.99	91
66					8.67	1.34	1.20	1.12	0.96	1.15	46
86					7.96	1.62	1.34	0.72	0.67	1.09	42
92					8.69	1.45	1.27	1.40		1.37	55
96					6.84	2.30	2.38	1.17	1.33	1.79	66
Mean						2.23	2.00	1.79	1.78	1.95	54
Maximum										6.81	
Minimum										0.67	

The loss in patients with hypertension was essentially the same, therefore, as in normal subjects. The senile subjects, on the other hand, lost much less. In all groups the loss from the toe tips was practically one-half that from the

finger tips; the variations were from 32 to 99 per cent but were much less in the senile groups.

TABLE II

Rate of Sweating (Mg./Sq. Cm. Skin Area/15 Min.) Measured Simultaneously on the Right Index Finger Tip and Right Second Toe Tip of 10 White, Resting Adults with Essential Hypertension

Subject no.	Date	Age (years)	Sex	Blood pressure (mm. Hg)	Area of skin (sq. cm.)	First 15 min.	Second 15 min.	Third 15 min.	Fourth 15 min.	Mean 15 min.	Rate of sweating as % of finger rate
Finger tips											
28	3/21/41	29	F	245/145	8.33	3.78	5.20	4.35	3.31	4.16	
37	4/ 3/41	37	M	230/130	10.73	4.40	8.70	8.01	8.80	7.48	
40	3/26/41	38	F	220/148	9.31	1.84	1.99	1.98	1.92	1.93	
44	3/27/41	43	M	198/140	14.49	3.04	2.53	2.12	1.75	2.36	
58	3/26/41	27	F	242/130	8.67	4.81	3.56	4.65	3.71	4.19	
70	3/24/41	54	F	230/120	9.51	1.85	2.00	1.89	1.80	1.89	
71	3/28/41	40	M	230/130	12.38	4.48	6.52	2.67	5.89	4.89	
72	3/27/41	28	F	260/160	8.08	4.60	2.97	3.22	3.75	3.64	
88	3/31/41	38	F	240/120	8.69	4.12	3.25	3.49	2.77	3.41	
97	3/24/41	56	F	256/110	9.85	2.58	2.57	2.27	2.05	2.37	
Mean						3.55	3.92	3.47	3.58	3.63	
Maximum										8.80	
Minimum										1.75	
Toe tips											
28					8.41	3.82	3.47	3.54		3.61	87
37					8.69	6.12	5.93	5.43	4.80	5.57	74
40					7.36	1.60	1.47	1.52	1.96	1.92	99
44					11.36	0.92	0.83	0.79	0.62	0.75	32
58					7.62	3.29	2.86	3.27	3.21	3.15	75
70					7.61	1.43	1.17	1.31	1.05	1.25	66
71					8.49	3.24	3.65	3.69	2.85	3.36	69
72					8.02	2.31	1.31	1.38	1.35	1.58	43
88					8.02	1.75	1.60	1.38	1.50	1.56	51
97					8.60	0.92	0.85	0.70	0.70	0.79	33
Mean						2.54	2.31	2.30	2.00	2.29	63
Maximum										6.12	
Minimum										0.62	

There were marked variations in the rate of sweating from person to person, and from fifteen-minute period to fifteen-minute period in the same person. The variation from period to period was greatest in normal and hypertensive

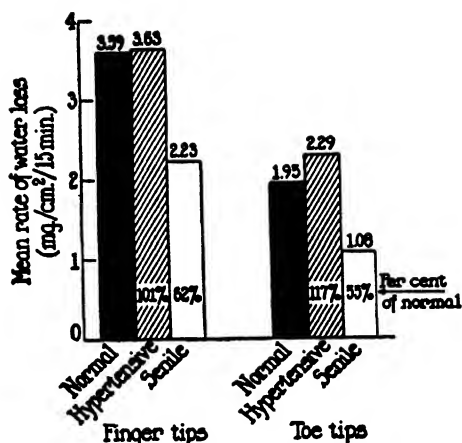


FIG. 1. Mean rates of sweating of the tip of the right index finger and right second toe of groups of resting normal, hypertensive, and senile subjects.

TABLE III

Rate of Sweating (Mg./Sq. Cm. Skin Area/15 Min.) Measured Simultaneously on the Right Index Finger Tip and Right Second Toe Tip of 8 Senile, White, Resting Adults

Subject no.	Date	Age (years)	Sex	Blood pressure (mm. Hg)	Area of skin (sq. cm.)	First 15 min.	Second 15 min.	Third 15 min.	Fourth 15 min.	Mean 15 min.	Rate of sweating as % of finger rate
Finger tips											
22	4/ 2/41	78	M	168/88	11.41	2.12	2.68	2.52	2.48	2.45	
27	4/ 3/41	78	M	132/84	10.38	1.85	1.78	1.90	1.97	1.88	
41	4/ 1/41	71	M	110/60	11.14	2.01	2.12	2.03	2.18	2.09	
47	4/ 1/41	79	M	130/72	10.70	2.73	2.34	2.34	2.25	2.41	
63	4/ 3/41	85	M	148/80	11.28	2.45	1.81	2.35	2.00	2.15	
69	3/25/41	75	M	148/70	10.79	2.44	2.39	1.71	2.87	2.35	
93	4/ 2/41	81	M	140/64	9.83	2.33	2.22	2.13	2.07	2.19	
95	3/25/41	70	M	136/80	10.39	2.08	2.61	2.34	2.33	2.34	
Mean						2.25	2.24	2.17	2.27	2.23	
Maximum										2.87	
Minimum										1.71	
Toe tips											
22					7.32	0.96	0.87	1.30	0.70	0.96	39
27					9.22	1.37	1.17	1.11	1.25	1.22	65
41					7.92	1.26	1.14	1.14	1.01	1.14	55
47					9.15	1.24	1.17	1.03	0.98	1.11	47
63					9.56	0.83	0.78	0.68	0.72	0.75	35
69					8.30	1.55	1.19	1.43		1.39	59
93					8.61	1.35	1.18	0.85	1.08	1.12	51
95					8.71	1.23	1.19	0.88	1.03	1.09	47
Mean						1.22	1.09	1.05	0.97	1.08	48
Maximum										1.55	
Minimum										0.68	

subjects and least in the senile ones. In fact, the senile subjects were noteworthy for their constancy. In one of the subjects (Subject 86, Table IV) the mean rate of sweating of the finger tip was only three-fifths as rapid on one day as it was six days later, whereas in the toe tip it was only slightly less. The variations were always much greater in the fingers than in the toes. Similar results were obtained on Subject 96 (Table IV).

TABLE IV

Rate of Sweating (Mg./Sq. Cm. Skin Area/15 Min.) of Finger Tips (F) and Toe Tips (T) of Pairs of Normal, Resting Subjects, Studied Simultaneously

Subject no.	Date	Part	Area of skin (sq. cm.)	First 15 min.	Second 15 min.	Third 15 min.	Fourth 15 min.	Mean 15 min.
6 and 96	5/ 6/41	F	8.64	1.31	1.23	1.71	1.28	1.38
		T	8.60	1.86	0.70	1.17	1.05	1.20
		F	6.90	2.97	2.59	3.12	8.74	4.37
		T	6.84	1.64	1.75	2.19	3.01	2.15
14 and 86	5/ 7/41	F	7.56	4.03	3.66	2.76	2.74	3.30
		T	7.98	1.56	1.04	1.13	1.17	1.23
		F	7.62	3.33	2.93	2.23	1.84	2.58
		T	7.96	1.62	1.34	0.72	0.67	1.09
86 and 49	5/13/41	F	7.62	3.18	5.59	2.81	5.46	4.26
		T	7.96	1.42	1.46	1.09	0.89	1.22
		F	8.66	5.57	4.50	3.46	2.75	4.07
		T	8.44	2.71	2.30	1.67	1.34	2.00
54 and 96	5/16/41	F	9.08	8.37	7.02	7.16	5.54	7.02
		T	8.29	2.24	1.62	1.91	1.91	1.92
		F	6.90	4.35	2.78	3.52	3.43	3.52
		T	6.84	1.55	1.37	1.21	0.91	1.26

When normal subjects were studied simultaneously in pairs, the rate of water loss increased in the finger or toe, or both, of one, while it remained the same or actually decreased in the other (Table IV). The variations from part to part in each of the pairs of subjects were discordant. The rate of sweating of the right and left index fingers or the right and left second toes also varied discordantly (Table V).

In hypertensive patients whose lower thoracic and lumbar sympathetic nerves had been cut and the coeliac and aortic renal ganglia excised, a decrease of more than half the rate of water loss from the second toe and an increase of about half the rate from the index finger were found (Fig. 2). These differences were great enough for subjects and observers to detect by qualitative methods.

TABLE V

The Rate of Sweating (Mg./Sq. Cm. Skin Area/15 Min.) of Right and Left Index Finger Tips of Resting Subjects, Studied Simultaneously

Subject no.	Part	Area of skin (sq. cm.)	First 15 min.	Second 15 min.	Third 15 min.	Fourth 15 min.	Mean 15 min.	Sweating rate of left finger as % of right	Diagnosis
2	Right	11.22	2.48	3.23	2.91	2.78	2.85	-3	Normal
	Left	10.97	3.50	1.58	3.22	2.81	2.76		
3	Right	10.23	2.48	2.13	2.09	1.87	2.15	-15	Normal
	Left	10.63	2.25	1.67	1.60	1.80	1.83		
24	Right	7.56	2.39	4.72	5.22	6.42	4.69	-5	Normal
	Left	7.17	1.92	3.85	5.43	6.71	4.48		
31	Right	8.85	4.31	5.88	5.10	6.96	5.56	+2	Normal
	Left	8.81	6.92	5.51	5.37	4.95	5.69		
54	Right	9.08	5.81	5.30	5.15	5.59	5.47	+1	Normal
	Left	8.57	5.95	5.80	4.83	5.41	5.50		
37	Right	10.73	4.40	8.70	8.01	8.80	7.48	-2	Hypertension
	Left	10.73	6.13	6.80	8.00	8.51	7.36		
40	Right	9.31	1.84	1.99	1.98	1.92	1.93	+12	Hypertension
	Left	8.35	2.20	2.12	2.01	2.35	2.17		
58	Right	8.67	4.81	3.56	4.65	3.71	4.19	-12	Hypertension
	Left	7.95	3.77	4.35	3.60	2.97	3.67		
71	Right	12.38	4.08	2.96		2.60	3.36	+4	Hypertension
	Left	11.62	3.48	3.53		3.53	3.51		
72	Right	8.08	4.60	2.97	3.22	3.75	3.64	+20	Hypertension
	Left	7.46	5.25	3.47	3.67	5.16	4.38		
27	Right	10.38	1.85	1.78	1.90	1.97	1.88	+21	Senility
	Left	10.48	2.09	2.08	2.48	2.43	2.27		
41	Right	11.14	2.01	2.12	2.03	2.18	2.09	+8	Senility
	Left	10.77	2.46	1.95	2.37	2.25	2.26		
47	Right	10.70	2.73	2.34	2.34	2.25	2.41	+9	Senility
	Left	10.36	3.19	2.62	2.36	2.29	2.62		
93	Right	9.83	2.33	2.22	2.13	2.07	2.19	-15	Senility
	Left	9.20	1.46	1.86	2.47	1.70	1.87		
95	Right	10.39	2.08	2.61	2.34	2.33	2.34	+6	Senility
	Left	9.50	2.56	2.56	2.49	2.26	2.47		
Mean								±9 21 1	
Maximum									
Minimum									

There were great variations in the rate of sweating of the finger and toe tips of a normal subject when measurements were made continuously for 12.25

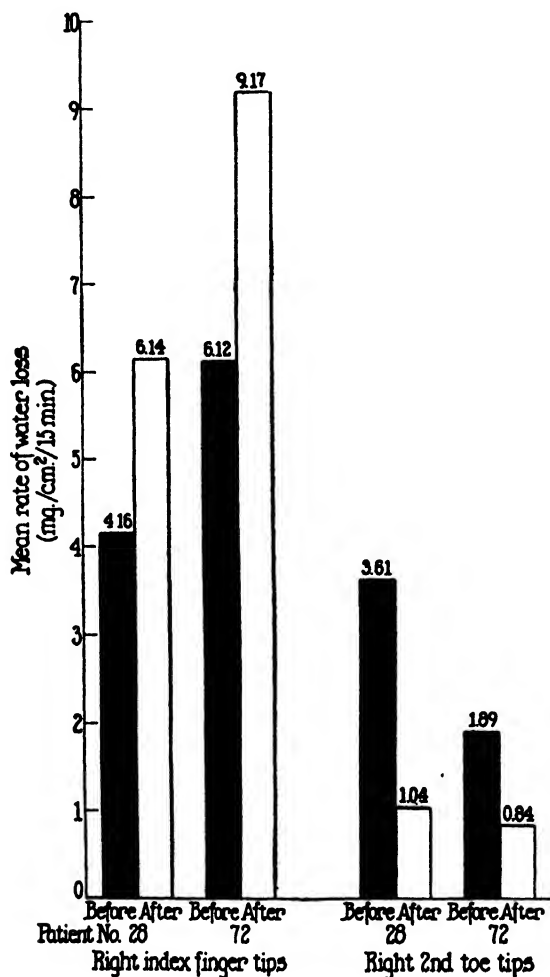


FIG. 2. The rate of sweating of the tips of the fingers and toes of two hypertensive subjects before, and three weeks after, bilateral sympathectomy (ninth thoracic sympathetic nerve to first lumbar and the celiac and aortic renal plexuses).

hours, with collections every fifteen minutes. (Fig. 3). Shortly after each meal (noon and evening), the rate increased for about ninety minutes. When the subject fell asleep, it reached a minimum which was very constant.

The rate of water loss of a patient with advanced Raynaud's disease and

scleroderma of the upper extremities whose cervical sympathetic nerves had been cut eight years before was 1.12 mg. in the right, and 1.25 mg. in the left, index finger tip per square centimeter per fifteen minutes. These rates were about one-third as rapid as in the same parts of normal subjects, and less than the normal minimum value. The rate of water loss from the right second toe tip was 1.85 mg. per square centimeter per fifteen minutes, a value similar to

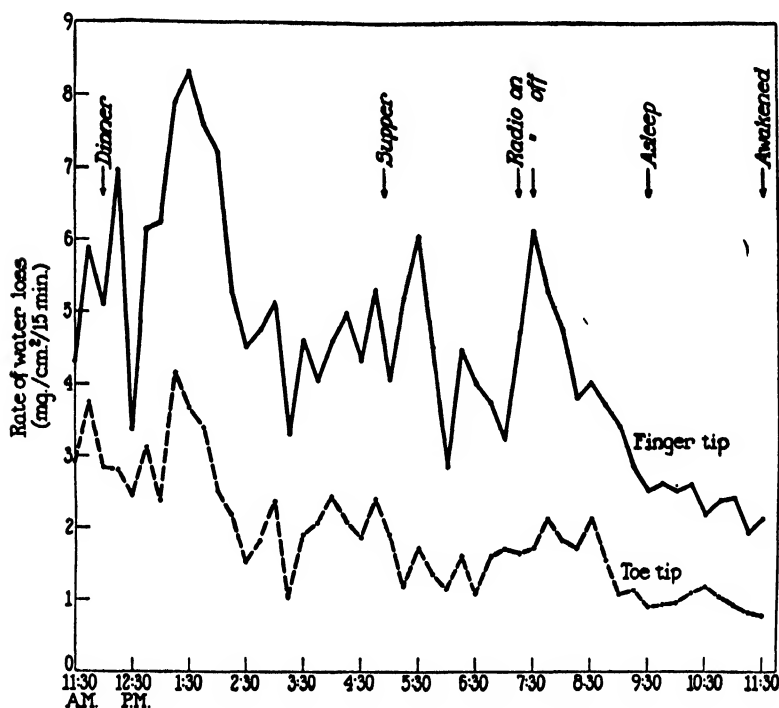


FIG. 3. Changes in the rate of sweating measured for fifteen-minute periods during an observation of 12.25 hours.

that of the normal group. Early Raynaud's disease of the toes was present in this patient.

DISCUSSION

The values for the rate of water loss from the tips of the fingers and toes of normal subjects are greater than in those previously reported.³ In the first series of normal persons the loss from the fingers was 2.10 mg. per square centimeter per fifteen minutes, and, from the toes, 1.42 (calculated for the area of the skin, not the total surface area), whereas, in the present series of normal subjects, it was 3.59 from the fingers and 1.95 from the toes. Many

of the subjects were studied in both series. The conditions were the same except for two factors: (1) The first study was conducted during the hot months of June, July, and August, 1940, and the present study, during the cool period of the last week of March, April, and the first two weeks of May, 1941. Although the temperature and humidity of the room were the same, subjects who entered the room during the hot summer months invariably commented on the coolness of the room, whereas those who entered this spring failed to make such comments. In fact, most of the subjects considered the room warm. That this is an important cause for the differences is not supported by the studies of Winslow, Herrington, and Gagge,⁵ who stated: "During the summer season, the mechanism of sweat secretion is in better working order than in winter so that the body responds to a given condition (in the zone of evaporation regulation) with a somewhat higher secretory activity." More extensive studies, over a period of a year, would, nevertheless, be necessary before a definite conclusion regarding seasonal influences could be reached. (2) In the first series, psychic disturbances played a role because of the presence of laboratory equipment in the same room with the subjects; this was markedly reduced in the second series.⁴ The importance of this factor could not be estimated, but it is true that many of the subjects were well acquainted with the laboratory and were not likely to be upset by it. There are many variables, however, in a person's daily and yearly activities that may be the basis of physiologic differences. The studies on patients were interspersed with those on the normal persons of the present series, and all observations were completed before the summer season began.

The marked variations in the same person from time to time and from one anatomic region to another at the same time were expected. The number of sweat glands which function at a given time is, as is well known, quite variable.^{5, 6} Sweating is influenced by many factors, including the psyche and fatigue, both of which lack uniformity, and both of which are always exerting some influence in any physiologic study on conscious persons.

In two subjects who were studied in the same room, in separate beds, under identical environmental conditions, there were discordant variations in their comparable anatomic parts (Table V). Such variations must be the result of conditions unrelated to the room. Furthermore, although the mean rate of sweating in the toes was always essentially one-half the mean rate in the fingers, this was not true for each individual measurement. The ratio of the rates of sweating in fingers and toes in any one subject varied markedly.

Under the conditions of our observations, the palmar surfaces of the hands were noticeably moist when the rate of water loss from the finger tip was 5 mg. or more.

The mean rate of sweating in the patients with hypertension was about the

same as that in the normal subjects. This fact is interpreted to mean that, under conditions of rest and quiet, the sympathetic tone, as indicated by its effect on sweating, is about the same in hypertensive as in normal persons. This is in keeping with the observations of others who studied the cardiovascular system and found no evidence of increased sympathetic tone.^{1, 2} Under conditions of stress and strain an increase may nevertheless exist.

The rate of sweating in the senile subjects was definitely less rapid and much less variable than in the normal persons. This is probably one of the general manifestations of reduced physiologic activity which are known to occur in senile persons. No attempt was made to learn what part this was of their total capacity to sweat. It is unknown whether the low rate of sweating is due to a decrease in the number of sweat glands, to partial atrophy, to decreased function, or to all three factors.

SUMMARY

The rate of water loss from the tip of the index finger and second toe of normal subjects was very variable. There were variations from one period of fifteen minutes to another, from finger to toe, from right finger to left finger, and from day to day in the same subject. The mean rate of sweating in the toe tips was approximately one-half that in the finger tips, although this ratio could be variable. The rate of sweating varied markedly from person to person under identical conditions of observation.

In the patients with arterial hypertension the rate and variations of water loss from the finger and toe tips were about the same as in the normal subjects. This suggests that, in resting persons with hypertension, sympathetic activity is no greater than in normal subjects.

In senile subjects the rate of water loss was much less than in the normal subjects and patients with hypertension. Furthermore, the rates from period to period were much less variable. In fact, the senile subjects were outstanding for the constancy with which they lost water. This may be part of the reduced physiologic activity which is known to occur in senile persons.

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COMPARISON OF SIMULTANEOUS INDIRECT (AUSCULTATORY) AND DIRECT (INTRA-ARTERIAL) MEASUREMENTS OF ARTERIAL PRESSURE IN MAN¹

By J. MURRAY STEELE, M.D.

*(From the Hospital of The Rockefeller Institute and the New York University
Medical Division of Welfare Hospital, New York)*

The accuracy of the indirect method of measuring arterial pressure in human beings by means of placing a pneumatic cuff about the upper arm and auscultating the sounds arising in the artery below it (1) has until recently been difficult to test. Application of Frank's principles of direct intra-arterial manometry (2, 3) to smaller and less sensitive manometers (4, 5) whose sensitivity is subsequently magnified by using longer beams of light, has permitted the use of cannulae for connecting artery and manometer of such small caliber that ordinary luer needles (18 to 25 gauge) may be employed. Finally, substitution of short pieces of flexible lead tubing for the older rigid metal ones to connect arterial cannulae to the manometers has made the positioning of subject and manometer so convenient that direct intra-arterial measurement of pressure in various arteries, often in two or three arteries simultaneously, of unanesthetized man and beast may be carried out without pain and with minimal disturbance. In individuals whose flesh is especially tender, arterial puncture may, in fact, often be performed with less discomfort than that which accompanies inflation of the cuff for measuring arterial pressure by the usual indirect technique.

In view of these advances in technique, it seemed desirable to compare measurements of arterial pressure obtained by the usual indirect technique with those obtained by an established direct method in order to furnish data for rational criteria for the use of the usual auscultatory technique. The instrument developed by Hamilton (4) was chosen.

Procedure. The patient is placed supine in bed, the arm supported horizontally at an angle of about 60° to the axis of the body on a small padded frame which supports the arm from elbow to wrist and at the same time allows clearance for inflation of the pneumatic cuff about the upper arm. The cuff is connected by the two arms of a Y-tube to a mercury manometer and to one of two Hamilton manometers. The needle of the second Hamilton manometer is inserted into the radial artery. The cuff is then inflated and deflated in the usual manner for estimation of arterial

¹ The latter part of this investigation has been aided by a grant from the Josiah Macy, Jr. Foundation.

pressure by listening for Korotkoff sounds with a stethoscope, while pressures in the radial artery and within the cuff are being simultaneously recorded by optical means. Note is made of the level of the mercury manometer at the appearance of sound, at the moment of sudden muffling and at the disappearance of sound (first, fourth and fifth phases respectively), and their occurrence is signalled on the photographic record.

With the help of these records, the systolic and diastolic pressures recorded by the Hamilton manometer may be compared with those estimated at the various phases with the help of Korotkoff's sounds by reading the level of the mercury manometer. The level of pressure in the pneumatic cuff may also be estimated for these points from the optical record, and thus serve as a guide to the correctness of the mercury manometer in indicating the pressure within the cuff. Observations obtained show that the effective size of the vent at the top of the mercury manometer is the factor which limits the speed at which the cuff can be deflated without lag in recording the pressure therein. With the manometers in general use, the pressure in the cuff may be lowered as rapidly as 8 mm. per second without significant loss of accuracy. It is, however, important to note that the distance in millimeters of mercury which the manometer drops between two beats contributes to error. This error is directly proportional to the rate of fall and inversely proportional to the pulse rate. Hence, a slow rate of fall (3 to 5 mm. per second) in passing the levels of systolic and diastolic pressure, is desirable. Although the differences between measurements of pressure in the cuff by the Hamilton and by the mercury manometer are small, they are significant and this phase of the problem is still under investigation.

The accuracy of the measurements obtained from the optical records of the direct manometer seems unquestionable in theory. Certain practical difficulties leave room for error. In order to avoid these errors so far as possible, the following precautions have been taken: 1) The frequency of the manometers has been tested regularly and records were not used unless the frequency was greater than 150 per second. 2) A needle of smaller caliber or of greater length than the one with which the manometer was calibrated was never used. 3) After insertion of the needle into an artery, it was ascertained that moderate changes in its position did not affect the character or amplitude of the pulse wave. 4) When the needle was withdrawn after the recording was complete, its point was examined for partial blocking by clot or by bits of tissue and, if present, the record was discarded. The patency of the needle was also ascertained by forcing citrate solution through it under pressure. A defect in the smooth ejection stream was readily observable.

Comparison between the two methods has been made 74 times in 39 individuals selected only in that they did not exhibit valvular defects or arrhythmias (Table I). Each comparison involves two separate successive

TABLE I

Comparison of direct and indirect arterial pressures in forty-one individuals are made. Direct manometer is in right radial artery. Standard pneumatic cuff (12 cm. wide), about right brachial artery.

PATIENT	SYSTOLIC PRESSURE MM. Hg			DIASTOLIC PRESSURES MM. Hg				
	Auscultatory	Direct manometer	Difference*	Auscultatory		Direct manometer	Differences*	
				4th phase	5th phase		4th phase	5th phase
1. E. K.	130	130	0	86	80	75	+11	+5
2. A. S.	124	128	-4	—	76	72	—	+4
3. R. B.	94	96	-2	68	60	58	+10	+2
4. R. S.	150	162	-12	80	70	74	+6	-4
5. R. S.	185	170	+15	114	110	112	+2	-2
6. C.	210	215	-5	—	130	132	—	-2
7. R. K.	156	164	-8	—	106	108	—	-2
8. H. S.	200	230	-30	140	130	130	+10	0
9. L. R.	234	255	-21	140	126	125	+15	+1
10. W. N.	214	242	-28	—	140	142	—	-2
11. E. T.	115	112	+3	75	69	70	+5	-1
12. A. S.	114	125	-11	80	76	82	-2	-8
13. A. S.	148	156	-8	—	80	73	—	+7
14. J. B.	115	119	-4	80	72	68	+12	+4
15. H. M.	241	242	-1	—	120	122	—	-2
16. J. W.	112	136	-24	—	84	78	—	+6
17. M. K.	152	192	-40	—	92	88	—	+4
18. W. J.	122	149	-27	90	80	80	+10	0
19. J. S.	210	212	-2	140	130	128	+12	+2
20. R. S.	130	146	-16	—	73	70	—	+3
21. T. R.	113	115	-2	67	60	62	+5	-2
22. D. S.	170	196	-26	—	92	90	—	+2
23. J. M.	142	126	+16	106	86	80	+26	+6
24. J. N.	120	125	-5	—	80	83	—	-3
25. T. R.	112	120	-9	—	61	58	—	+3
26. W. J.	114	135	-21	—	69	70	—	-1
27. J. B.	122	126	-4	70	68	62	+8	+6
28. R. S.	122	145	-23	68	60	52	+16	+8
29. S. S.	262	270	-8	—	164	164	—	0
30. L. C.	126	149	-23	—	78	80	—	-2
31. J. F.	160	159	+1	—	80	79	—	+1
32. L. H.	162	184	-22	88	84	89	-1	-5
33. A. B.	116	126	-10	74	68	68	+6	0
34. W. S.	129	131	-2	90	80	82	+8	-2
35. E. G.	131	138	-7	72	68	63	+9	+5
36. J. H.	144	170	-26	86	80	90	-4	-10
37. J. C.	163	156	+7	78	74	75	+3	-1
38. J. P.	144	146	-2	96	92	86	+10	+6
39. G. B.	150	152	-2	92	86	81	+11	+5
Average	150	160	-10	90.1	88	87.3	+8.8	+0.8
40. P. O.	138	150	-12	70	50	50	+20	0
41. J. D.	108	139	-31	70	45	67	+3	-22

* Differences are recorded with the direct record as a standard.

simultaneous measurements by each method. In ten individuals, two comparisons were made. The ages ranged from 22 to 86 years. Twenty-one were normal, seven suffered from arterial hypertension alone, three from hypertension and arteriosclerosis, and eight from arteriosclerosis alone. Two were quite obese. It is obvious that the number of patients in each group is, as yet, far too small to bring out clearly the limits of the indirect method. Although the data are not yet sufficient to permit describing rules for estimating arterial pressure by the indirect method of Korotkoff, they suggest that revision of the present empiric criteria (6) may be necessary when sufficient data have been obtained. Certain tendencies are of interest.

After the pulse wave in the record of arterial pressure from the radial artery has been obliterated by inflating the pneumatic cuff about the upper arm to a pressure above systolic level, pressure within the artery distal to it falls below the usual diastolic level. As the pressure in the cuff is lowered gradually, the first or second (fig. 1 A, B, C), rarely the third, beat (fig. 1 D) to become visible in the arterial record is audible; which one becomes audible appears to depend partly upon the speed of deflation and partly upon the form of the pulse wave. Of interest is the fact that the systolic level recorded by the Hamilton manometer immediately after release of the cuff is often elevated by 6 to 12 mm. Hg. for about 10 seconds and consequently the level during this period was not used for comparison.

The beat which is audible or visible usually occurs below the systolic level of pressure recorded in the radial artery by the Hamilton manometer (Table I and fig. 2). There does not appear to be any greater difference for high, than for low pressures. The average systolic level obtained by auscultatory technique is, for the present data, 10 mm. Hg. below that obtained by the intra-arterial measurements.

It seems to be generally believed that diastolic pressure is more difficult to record than systolic; but when the diastolic pressures taken by the auscultatory method are compared to those measured directly, the differences between the two appear to be less than those obtained in measurements of systolic pressure and of opposite sign. When the fifth phase (disappearance of sound) is used, the average difference between auscultatory and direct readings is negligible (less than 1 mm.). When the fourth is used, the auscultatory level averages 8.8 mm. higher than the direct. In only three instances was the diastolic pressure closer to the direct reading at the fourth phase, than that at the fifth (Table I, fig. 2). When muffling and disappearance of sound take place almost simultaneously, the single pressure has been recorded in the column labelled "fifth phase". The data indicate that the disappearance of sound, except in the case of aortic insufficiency, is a more accurate guide to diastolic pressure than the sudden muffling recommended by the English component of the Committee for Standardization of Blood Pressure Readings (6) and by Bramwell (7).

Two observations with regard to diastolic pressure in aortic insufficiency have been made. In one, the fifth phase of Korotkoff's sound is the better estimate of diastolic level (Case 40 and fig. 3) and in the other, the fourth (Case 41). Sound often persists, when this valvular lesion is present, until the cuff is completely deflated indicating a pressure of zero, which is obviously unlikely.

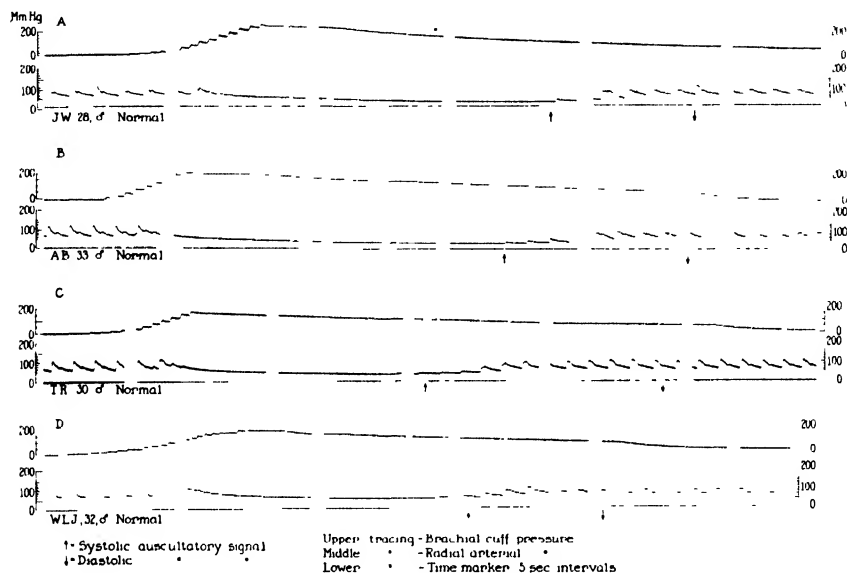


FIG. 1. Optical records of arterial pressures in the right radial arteries of four normal individuals during compression of the brachial artery by a pneumatic cuff are shown.

	DIRECT PRESSURES		AUSCULTATORY PRESSURES	
	Systolic	Diastolic	Systolic	Diastolic
A	125	80	112	84
B	116	68	126	68
C	115	62	113	60
D	122	90-80	119	78

The beat immediately preceding the arrow pointing upward is the first audible beat. Reduced to one-quarter the original size.

The divergence of the two methods in systolic pressure invites discussion. Part of the difference is easily accounted for by two well recognized facts. First, systolic pressure is higher in the radial than in the brachial artery. Woodbury, Murphey and Hamilton (8) gave differences of 8 and 9 mm. Hg in a normal individual and in a case of coarctation of the aorta respectively for the difference between the *axillary* and radial arteries. The difference

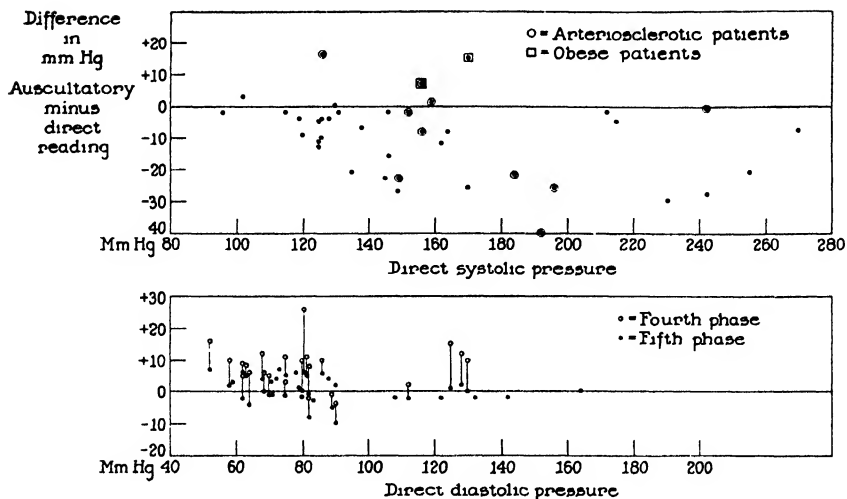


FIG. 2. The data contained in Table I are represented graphically. Instances in which the auscultatory measurements coincide with the direct, fall on the zero line. When a point falls below or is above the zero line, the vertical distance from the line represents the degree to which the auscultatory measurement either falls short of or exceeds the direct measurement.

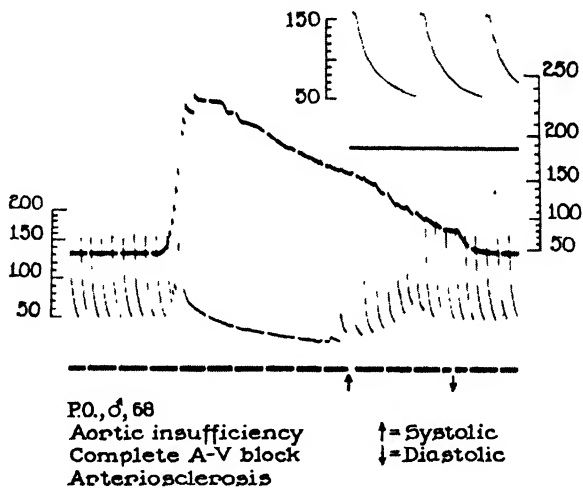


FIG. 3. Optical records of the movements of two Hamilton manometers are shown. The lower tracing records arterial pressure in the right radial artery (left hand scale); the upper tracing, pressure changes in the pneumatic cuff around the brachial artery while it is being inflated and deflated (right hand scale). *Direct* pressure levels recorded are systolic 150; diastolic 52 mm. Hg; *auscultatory* pressure levels are systolic 148; diastolic, 4th phase, 70, 5th phase, 50 mm. Hg.

should be less between the *brachial* and radial arteries. In the one instance in which the author has measured this difference it amounted to 5 mm. Hg. Secondly, unless the first beat at systolic level is heard, an additional error

in the same direction will be made, an error which depends on the rate of the pulse and the speed with which the mercury column is falling. In the present studies, this error probably varied from 3 to 6 mm. Hg. A correction for these two factors would account for the observed differences in roughly half of the cases (those below 10 mm. Hg. difference).

Concerning the larger differences, Robinow, Hamilton, Woodbury and Volpitto (9) and Bordley (10) have suggested that the form of the pulse wave may have a bearing upon the question. Those with sharp peaks, a form which suggests small changes in volume at high pressures, are the ones which tend to give lower auscultatory readings in relation to the direct readings. Bordley suggests that the pressure wave resulting from the

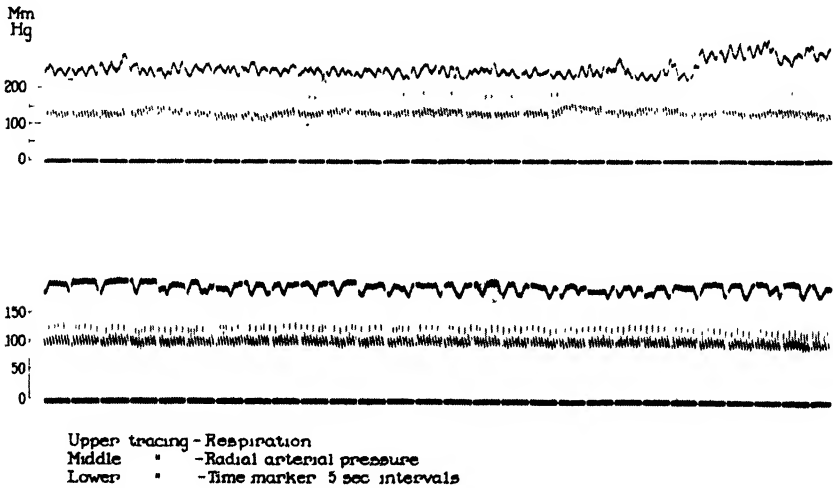


FIG. 4. Optical records of respiration and arterial pressure in the radial arteries of two elderly individuals while lying quietly during a long study are shown. The portions selected are representative fractions of continuous tracings obtained over periods lasting for more than twenty minutes to show changes in levels of pressure, some of which appear to be synchronous with respiratory rhythm and some of which do not.

smaller volume may be more readily damped in passage under the cuff. The present data fit in, for the most part, (fig. 1 A and B compared with C) with this view, but exceptions occur. Two of the six instances in which systolic pressure was, contrary to the usual experience, estimated to be at a higher level by the auscultatory than by the direct method, were in extremely adipose individuals. The relation between size of cuff and size of arm in these cases deserves careful study and involves diastolic as well as systolic pressures. Observations in several individuals during a long period of loss of weight (11) tend to show that larger cuffs do, in some obese people, give lower readings for arterial pressure than the standard cuff yields and more nearly approximate the levels obtained with direct measurement.

The other four observations in which the auscultatory technique yielded a higher pressure than the direct, occurred in individuals with arteriosclerosis, and yet arteriosclerosis was also present to a marked degree in at least four individuals in whom the error was large in the usual direction (fig. 2). An additional obvious reason for discrepancy between the two methods is the continual variation of both systolic and diastolic pressure not only with respiration (fig. 4) but also independently of respiration. These latter variations may correspond to the phasic changes in blood flow described by Burch, et al. (12) or to Traube-Hering waves. Whatever the reason for the change in level, it is clear that the indirect method singles out one beat, the first one to force its way through the cuff, while the direct method records a succession of beats. Measurements of the range of variability of arterial pressure are being carried out and will probably help to explain in part the differences between the methods but cannot do so entirely for the reason that the order of magnitude of the spontaneous variations is usually only from 5 to 10 mm. Hg.

In the case of diastolic pressure, the systematic discrepancies due to difference in site of measurement do not occur. Woodbury, Murphey and Hamilton's measurements (8) as well as my own observations failed to show significant differences in diastolic pressure along the course of the brachio-radial artery. In addition, respiratory and other variations of diastolic pressure are less with the result that direct and indirect diastolic measurements agree more closely.

SUMMARY

Concerning the comparison of simultaneous measurements of arterial pressure obtained by direct intra-arterial manometry and by indirect auscultatory technique in 39 individuals, it may be said that:

1. Systolic pressure was underestimated in indirect measurement by about 10 mm. Hg. In the present study, the indirect pressure in the brachial was compared with the direct pressure in the radial artery. This procedure may account for half of this difference.

2. In auscultatory technique the disappearance of sound proved to be a more accurate measure of diastolic pressure than the sudden muffling. The former over-estimated diastolic pressure by 8.8 mm. Hg., the latter by less than one.

3. The indirect auscultatory method of estimating arterial pressure is, considering its convenience and simplicity, an unusually accurate bedside method.

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THE SPECTROSCOPIC STUDY OF BIOLOGICAL EXTRACTS

I. URINE*

By K. DOBRINER, M.D., G. I. LAVIN, PH.D., AND C. P. RHOADS, M.D.

(From the Memorial Hospital for the Treatment of Cancer and Allied Diseases and The Hospital of The Rockefeller Institute for Medical Research)

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Increased interest in the metabolism of aromatic and cyclic hydrocarbons has made necessary the application of improved methods for the detection of minute amounts of these compounds in biological extracts. In metabolic studies only limited quantities of starting material are usually available and the amounts of certain metabolites present are too small to permit the isolation of pure compounds. Colorimetric, polarographic, fluorescence, and spectroscopic methods have been employed for the detection of these compounds, but the results have not been entirely satisfactory in all instances.

Spectroscopic procedures have been applied to the study of the constituents of biological materials, such as urine and blood (24, 26, 34, 39, 46, 55, 57, 63, 78, 101, 104), because many aromatic compounds isolated from these sources have specific absorption bands in the ultraviolet region of the spectrum. In most instances in the past, however, the spectroscopic studies have been carried out on unpurified material and the results have been somewhat unsatisfactory for the following reasons: (a) The materials studied contained a mixture of many aromatic compounds which gave overlapping absorption bands; hence, no specific and detailed bands suitable for the identification of substances in mixtures were observed. (b) The presence of a relatively large amount of a compound with a low extinction coefficient, or a small amount of a compound with a high extinction coefficient, interfered with the identification of other substances with a comparatively low extinction coefficient. (c) The presence of material responsible for a continuous absorption decreased the sharpness of individual bands and shifted them, thus making the interpretation difficult or uncertain.

Despite these disadvantages, spectroscopy provides in many instances more specific and detailed information in regard to the compounds under consideration than do colorimetric reactions. Because of the unsatisfactory results of previous work on untreated biological material (24, 26, 34, 39, 45, 46, 55, 57, 63, 78, 101, 104), a method of preliminary chemical fractionation of the ether-soluble substances of urines was employed by which the various absorbing compounds in a mixture could be separated from each other. At the outset

* This investigation was aided by grants from The Rockefeller Foundation and from The Jane Coffin Childs Memorial Fund for Medical Research.

it was necessary, therefore, to determine to what extent such fractionation procedures must be carried out in order to detect spectroscopically various aromatic substances in mixtures.

Material

Only studies on the urine of human beings are reported in this communication. Twenty-four hour specimens of urine were collected from 48 different individuals. Of these, 10 were normal, 26 were patients with pernicious anemia, refractory anemia, or leukemia, and 12 were pregnant women.¹ Several collections from the same individual were usually investigated.

METHODS

I. Spectroscopic Procedures

The spectrograph was a small quartz instrument manufactured by Hilger (Model No. 484), taking a $3\frac{1}{4}$ by $4\frac{1}{4}$ inch plate. The light source employed was an all-quartz hydrogen discharge tube (manufactured by the Hanovia Chemical Co.). In order to bring out all the absorption bands, a quartz Baly cell, with a micrometer adjustment, was employed. To compare the absorption bands of an unknown with those of a known substance, one plate was superimposed upon the other against a suitable light source. The hydrogen lines in the region of 4,800 Å. were used as reference lines. A few plates were studied with a microphotometer and the tracings can be seen in Fig. 18. For the quantitative measurements a Spekker spectrophotometer with a condensed spark as the light source was used (Fig. 17).

II. Fractionation of Urine

Acidified urines, both hydrolyzed and unhydrolyzed, were extracted with ether,² and the ethereal extracts separated into fractions containing the acidic, phenolic, and neutral ether-soluble substances. The acidic and phenolic compounds were further separated by steam distillation into their steam-volatile and nonsteam-volatile components. An outline of the procedures of extraction and separation is presented in Fig. 1.

*A. Preparation, hydrolysis, and ether extraction of urine (AEI and BEI fractions).—*The urine excreted during a 24-hour period was collected with a few cc. of chloroform as a preservative and was kept in the refrigerator at a

¹ The urine of pregnancy was obtained through the courtesy of Dr. Herbert Traut of the New York Hospital.

² Mallinckrodt reagent ether was used. On several occasions it was noticed that after the ether had been concentrated 10 to 20 times a number of absorption bands were found. These were in the region of 2,700 Å. and 3,000 Å. For this reason it was necessary to examine each container of ether.

temperature of 4°C. The patients received no medication of any kind for 3 days before, as well as during, the collection period, since derivatives of certain drugs excreted in the urine may give absorption bands. Concentrated hydrochloric acid was added to the urine to bring the pH to about 1.0. The urine was subsequently divided into equal parts designated as A and B. Part A was extracted with ether without further treatment, and part B was hydrolyzed by autoclaving for 2 hours at a pressure of 15 pounds and a temperature of 115°C. (6, 14, 75, 89, 94, 97). Part B was then extracted with ether. In a number of experiments, the total acidified urine was extracted with ether. The urine residue was then hydrolyzed by autoclaving and was subsequently extracted with ether. Fractions extracted before hydrolysis are designated by the letters AE, while ethereal fractions from hydrolyzed urine are designated by the letters BE. Extractions were made continuously for 24 hours in a Kutscher-Steudel apparatus which provided an even distribution of the ether through a sintered glass disc. For the spectroscopic survey the ethereal fractions were concentrated on the steam bath to a volume of 10 to 50 cc.

B. Separation of the ether-soluble substances into acidic, phenolic, and neutral components. 1. (a) *Fraction containing the acidic substances (AE3 and BE3).*—The ether extracts (AE1 and BE1) containing the total ether-soluble substances were brought to a volume of roughly 150 cc., and were extracted 3 times with 30 cc. of a solution of 10 per cent sodium carbonate. These combined sodium carbonate solutions were extracted 3 times with 150 cc. of ether, and all the ether extracts were combined to give the AE2 and BE2 fractions containing both the phenolic and the neutral substances. The sodium carbonate containing the acid substance was acidified with hydrochloric acid, extracted 3 times with equal volumes of ether, and the ethers combined to give the AE3 and BE3 fractions.

(b) *Separation of the steam-volatile from the nonsteam-volatile acidic substances (AE8 and AE9, and BE8 and BE9).*—Steam distillation was carried out in an all-glass apparatus of the Kjeldahl type fitted with an effective water-cooled condenser. The ether (AE3 and BE3) containing the acidic compounds was transferred to the Kjeldahl flask, 2 cc. of 10 per cent sodium hydroxide solution were added, and the ether was evaporated on the water bath. The fraction was then acidified with 5 cc. of 20 per cent hydrochloric acid solution, and steam-distilled until approximately 500 cc. of distillate was obtained, while the volume of the fluid in the flask was kept under 20 cc. Both the volatile material in the distillate and the nonvolatile material in the flask were made strongly acid with hydrochloric acid and extracted 3 times with equal volumes of ether. The ether extract containing the nonsteam-volatile material (AE8 and BE8) was concentrated on a steam bath to a volume of 10 cc., and that containing the steam-volatile material (AE9 and BE9) to a volume of 25 cc.

2. (a) *Fraction containing the phenolic substances (AE5 and BE5).*—The

ether (AE2 and BE2) remaining after the sodium carbonate extraction was brought to a volume of approximately 150 cc. and was extracted 3 times with 30 cc. of 20 per cent sodium hydroxide solution. Subsequent steps were carried out exactly as has been described for the acid compounds. The final

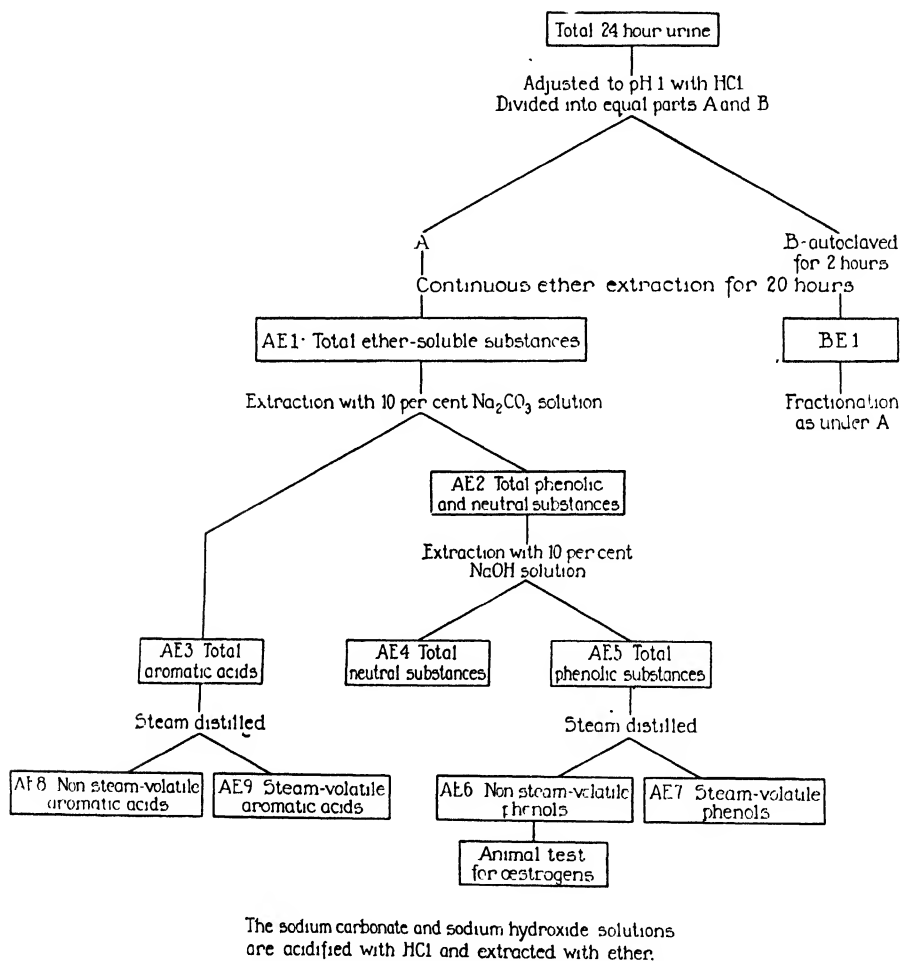


FIG. 1. Scheme for the separation of the ether-soluble substances from urine

etheral extract (AE5 and BE5) contained the phenolic compounds. The ether (AE4 and BE4) remaining after the sodium hydroxide extraction contained the neutral substances.

(b) *Separation of the steam-volatile (AE7 and BE7) from the nonsteam-volatile (AE6 and BE6) phenolic compounds.*—The fractions were prepared as described under B, 1(b).

3. *Fraction containing the neutral substances (AE4 and BE4).*—The ether remaining after extraction with sodium hydroxide (AE4 and BE4) was washed with 5 per cent hydrochloric acid and the ether was concentrated on a steam bath to a volume of 25 cc. This fraction contained the neutral substances.

RESULTS

A. Experiments to Test the Completeness of the Fractionation Procedure

A series of experiments with mixtures of pure chemical compounds was made to establish the adequacy of the method employed for fractionation. A complete separation of a mixture of the pure aromatic substances found in urine, as well as their spectroscopic differentiation, was necessary before any spectroscopic survey of fractions of urine could be undertaken.

Experiment 1.—The first experiment was made to ascertain whether the steam-volatile, acidic, and phenolic compounds could be distilled quantitatively. Two different mixtures of steam-volatile substances, which are known to be present in urines, were worked up as described under fractionation methods in II B. These mixtures were as follows: 1. mixture of benzoic acid (30 mgm.) and phenylacetic acid (70 mgm.); 2. mixture of phenol (10 mgm.) and *p*-cresol (90 mgm.).

The absorption bands given by the steam-volatile fraction were in the same positions as those given by the mixture of the pure substances. The fractions which should contain the nonsteam-volatile substances did not show any absorption bands, a fact which indicates that the distillation was complete as concerns both groups of substances.

Experiment 2.—A second experiment was made to test whether the nonsteam-volatile acidic and phenolic compounds would remain quantitatively in the nonsteam-volatile fraction. The following solutions containing pure substances were submitted to the fractionation procedure described under II B: 1. mixture of indolacetic acid (30 mgm.) and *p*-hydroxyphenylacetic acid (100 mgm.); 2. estriol (100 mgm.).

In the fraction which should contain the steam-volatile compounds no absorption bands were seen. In the fractions containing the nonsteam-volatile substances the absorption bands were in the same positions as the bands given by the pure compounds. Judged by the sensitivity of the spectroscopic method as applied, the separation was complete.

Experiment 3.—In a third experiment the following mixture, consisting of 8 pure neutral, phenolic, and acidic substances, was submitted to the complete chemical fractionation procedure as shown in Fig. 2: 1. neutral material,—indigo red (100 mgm.); 2. phenolic material,—phenol (10 mgm.), *p*-cresol (90 mgm.), estriol (100 mgm.); 3. acidic material,—benzoic acid (30 mgm.), phenylacetic acid (70 mgm.), indolacetic acid (30 mgm.), and *p*-hydroxyphenylacetic acid (100 mgm.).

As seen in Fig. 2, a separation of the compounds into fractions suitable for

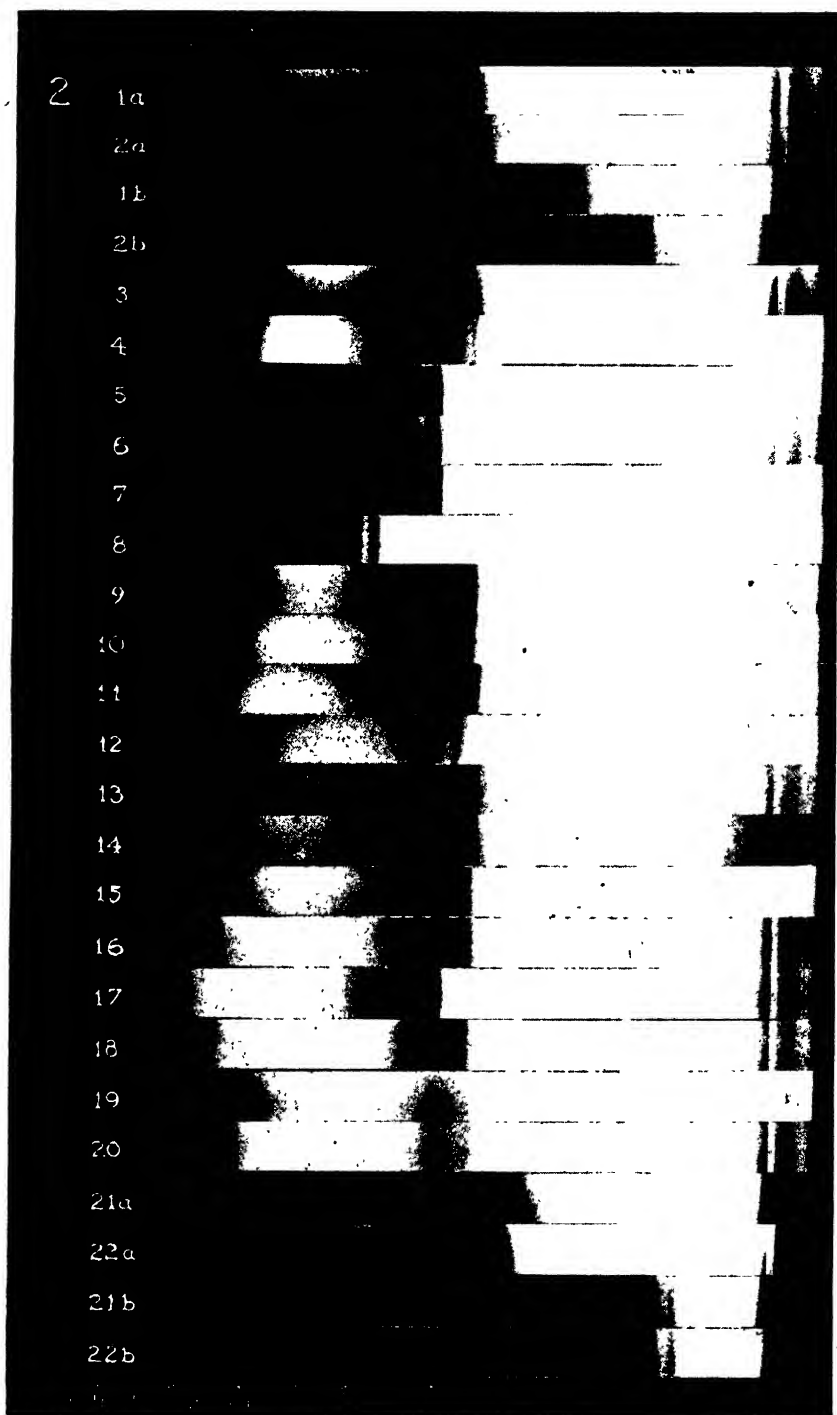


FIG. 2

spectroscopic differentiation has been obtained. Identical solutions were made up artificially from pure compounds or mixtures of pure compounds. A comparison of the bands from the fractions and from the artificial solutions demonstrates that the bands given by the fractionated compounds or mixtures of compounds occur in the expected fraction and are identical with the bands of the artificial mixtures.

It can be seen from Fig. 2 that in fraction AE1, containing a mixture of 8 different substances, no differentiation of the individual components is possible. In fraction AE2, overlapping of bands prevents the differentiation of the 4 substances present. In fraction AE3, with 3 compounds present, the estriol cannot be differentiated. On the other hand, in fractions AE8, AE6, AE7, and AE4, absorption bands characteristic of all the 8 individual substances present in the mixture can be accounted for.

B. Spectroscopic Survey of Urines

1. General Discussion of the Ether-Soluble Aromatic Acids of Urine

Certain aromatic acids, products of the intermediate metabolism of aromatic amino acids and of the bacterial decomposition of proteins in the intestinal tract, are present in urine excreted by both normal and diseased individuals. The acids are all derivatives of phenylalanine, tyrosine, or tryptophane:

a. Phenylalanine derivatives:

1. Benzoic acid (2, 22, 30, 47, 59, 76, 89, 96).
2. Phenylacetic acid and phenylpropionic acid (1, 12, 31, 49, 76, 80, 82-84, 87-89, 98, 99, 102).
3. Phenylpyruvic acid and phenyllactic acid (25, 38, 48, 52, 70, 77).

b. Tyrosine derivatives:

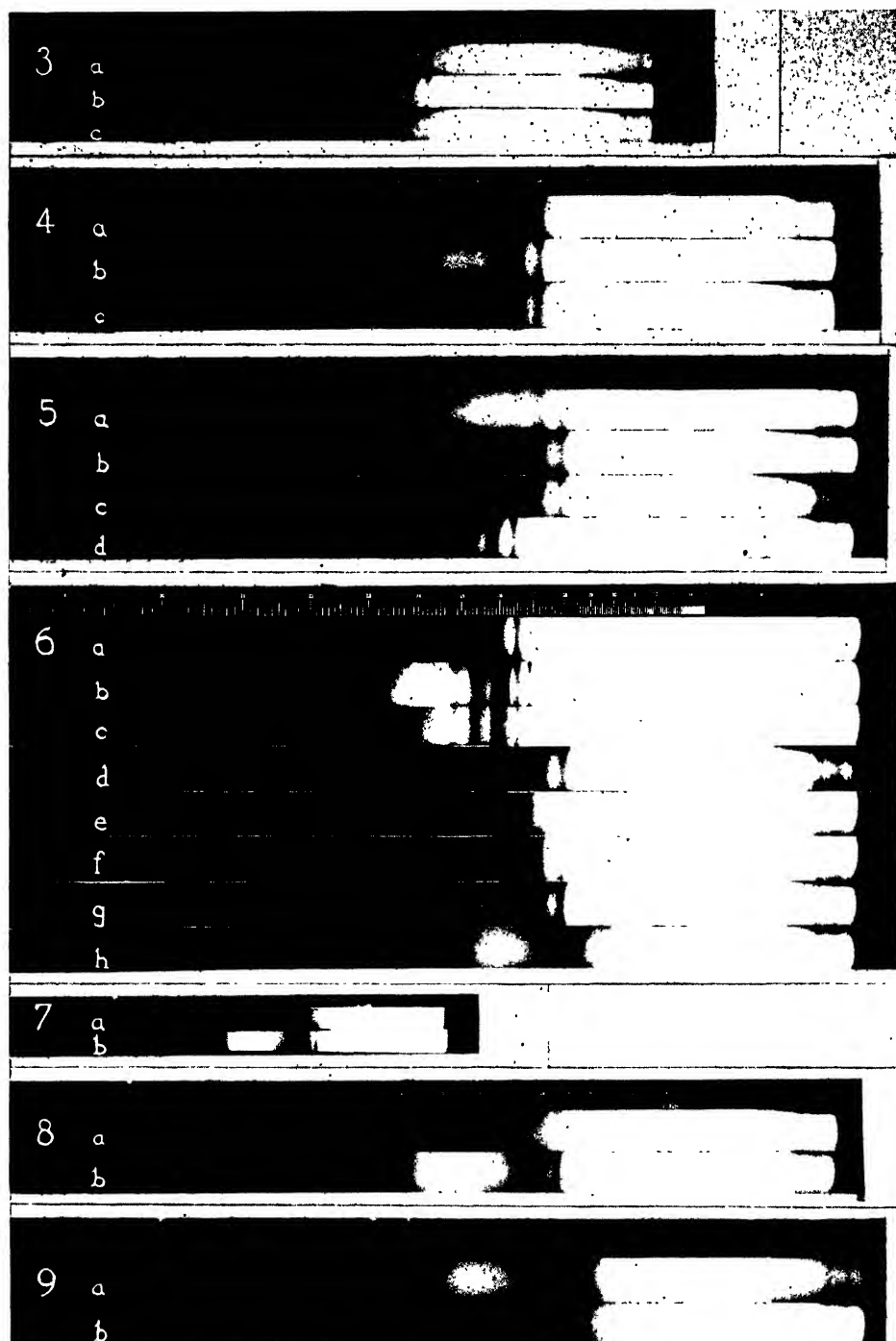
1. *p*-Hydroxybenzoic acid (6, 14, 75, 89, 94, 97).
2. *p*-Hydroxyphenylacetic acid and *p*-hydroxyphenylpropionic acid (2, 3, 5, 10, 11, 16, 17, 56, 58, 89, 91, 97).
3. *p*-Hydroxyphenylpyruvic acid and *p*-hydroxyphenyllactic acid (3, 17, 35, 40, 53, 54, 67, 95).

c. Tryptophane derivatives:

1. Indol-3-acetic acid and indol-3-propionic acid (43, 44, 51, 85, 86).

DESCRIPTION OF FIGURE 2

FIG. 2. Absorption spectrograms of artificial mixtures of acidic, phenolic, and neutral aromatic substances. (1a)—AE1, low concentration. (2a)—AE2, low concentration. (1b)—AE1, high concentration. (2b)—AE2, high concentration. (3)—AE3. (4)—Mixture of benzoic acid, phenylacetic acid, indolacetic acid, and *p*-hydroxyphenylacetic acid. (5)—AE9. (6)—Mixture of benzoic acid and phenylacetic acid. (7)—Benzoic acid. (8)—Phenylacetic acid. (9)—AE8. (10)—Mixture of indolacetic acid and *p*-hydroxyphenylacetic acid. (11)—Indolacetic acid. (12)—*p*-Hydroxyphenylacetic acid. (13)—AE5. (14)—Mixture of phenol, *p*-cresol, and estradiol. (15)—AE7. (16)—Mixture of phenol and *p*-cresol. (17)—Phenol. (18)—*p*-Cresol. (19)—AE6. (20)—Estradiol. (21a and b)—AE4. (22a and b)—Indigo red.



FIGS. 3 TO 9

A partial separation of these substances can be made by steam distillation, since the substances of groups a1 and a2 are steam-volatile while groups a3, b, and c are not steam-volatile.

The aromatic acids are present in urine partly in the free form but are principally conjugated with glucuronic acid, sulfate, glycocholic, or glutamine. The free compounds are readily soluble in ether, whereas the conjugated compounds are practically insoluble. The chemical isolation and identification of amino acid derivatives, with the exception of benzoic acid, is not practicable from 24-hour urine collections. Without actual isolation of compounds nonspecific tests must be employed (71). This is especially true for the oxidized aromatic compounds which give the same color reactions as do the phenols.

II. Results of Spectroscopic Survey of the Ether-Soluble Aromatic Acids of Urine

a. The total ether-soluble aromatic acids (Fig. 3) (AE3 and BE3).—This fraction gave a strong general absorption which decreased the intensity of the bands which were present. Furthermore, a mixture of substances with overlapping bands was present which made the differentiation of individual compounds difficult, if not impossible.

In (a) of Fig. 3 are shown the absorption bands given by the acidic ether-soluble substances from hydrolyzed urine (BE3); in (b) the bands given by the steam-volatile compounds of the same fraction (BE9); and in (c) the bands given by the nonsteam-volatile substances (BE8).

Comparison of the bands demonstrates that much sharper and more detailed

DESCRIPTION OF FIGURES 3 TO 9

FIG. 3. (a)—Total aromatic acids from hydrolyzed urine (BE3). (b)—Steam-volatile constituents from (a) (BE9). (c)—Nonsteam-volatile constituents from (a) (BE8).

FIG. 4. (a)—Steam-volatile aromatic acids from unhydrolyzed urine (AE9). (b)—Benzoic acid. (c)—Hippuric acid.

FIG. 5. (a) Benzoic acid. (b)—Steam-volatile aromatic acids from hydrolyzed urine (BE9). (c) Mixture of benzoic and phenylacetic acids. (d)—Phenylacetic acid.

FIG. 6. (a)—Phenylacetic acid. (b)—Phenylpropionic acid. (c)—Mixture of phenylacetic acid and phenylpropionic acid (1:1). (d)—Mixture of phenylacetic acid, phenylpropionic acid, and benzoic acid (3 parts of a mixture of 1:1 phenylacetic and phenylpropionic acid and 1 part of benzoic acid). (e)—Mixture of benzoic and phenylpropionic acids (1:3). (f)—Mixture of benzoic and phenylacetic acid (1:3). (g)—Benzoic acid. (h)—Phenylpyruvic acid.

FIG. 7. (a)—Nonsteam-volatile aromatic acids from unhydrolyzed urine (AE8). (b)—Indol-3-acetic acid.

FIG. 8. (a)—Nonsteam-volatile aromatic acids from hydrolyzed urine (BE8). (b)—*p*-Hydroxyphenylacetic acid.

FIG. 9. (a)—Mixture of indol-3-acetic and *p*-hydroxyphenylacetic acids (b)—Nonsteam-volatile aromatic acids from unhydrolyzed urine (AE8).

bands were obtained after steam distillation since that procedure largely separated groups of compounds which gave overlapping absorption bands. A considerable amount of coloring material giving a background absorption remains in the solution after distillation, since it then becomes ether-insoluble.

b. Steam-volatile, ether-soluble aromatic acids. I. Fraction obtained from unhydrolyzed urine.—Benzoic acid (AE9) Fig. 4. Absorption bands were present in this fraction (4a) at positions identical with those given by benzoic acid. This compound was liberated, presumably, by the hydrolysis of hippuric acid during the steam distillation of the (A) fraction. In many instances the bands given by this fraction from unhydrolyzed urine were identical with those given by a similar fraction obtained after hydrolysis, but the concentration of benzoic acid was greater in the latter.

For comparison the bands given by hippuric acid are shown in Fig. 4(c). These bands are similar to, but slightly different in position from those given by benzoic acid (b). The shift is so slight that it is only noticeable in the original plates and not in the reproductions.

2. Fraction obtained from hydrolyzed urine.—Benzoic acid and phenylacetic acid (BE9) Fig. 5. This fraction from urine (b) showed a series of bands similar to those given by a mixture of benzoic and phenylacetic acids (c). In Fig. 5a the absorption of benzoic acid and in Fig. 5d that of phenylacetic acid is shown. In the urines of normal, as well as of diseased individuals, there were considerable variations in the relative amounts of these compounds.

Although both phenylacetic acid and phenylpropionic acid may occasionally be present in urine (1, 12, 31, 49, 76, 80, 82-84, 87-89, 98, 99, 102), one cannot differentiate, spectroscopically, the latter (Fig. 6c) in the presence of the former (Fig. 6a) because of the slight difference in the absorption of these homologues. In illustration of this point, the absorption bands of pure phenylpropionic acid alone are compared with those of a mixture of pure phenylpropionic acid and phenylacetic acid (Fig. 6b and 6c). In the urines so far investigated, the absorption bands characteristic of phenylpyruvic (Fig. 6h) and phenyllactic acids have not been observed. The bands given by some of these acids and their mixtures are shown in Fig. 6.

c. The nonsteam-volatile, ether-soluble, aromatic acids (AE8 and BE8). I. Indol-3-acetic acid (Fig. 7).³—In fractions of urines from both healthy and diseased individuals bands were occasionally present in positions similar to those of indol-3-acetic acid (Fig. 7). The bands were present only in the fractions from unhydrolyzed urine, since acid hydrolysis transforms indol derivatives.

Indol-3-propionic acid gives the same series of bands as indol-3-acetic acid except that the bands are slightly shifted towards the longer wave lengths.

³ Most of the photographs shown in this paper are plates of enlarged two diameters. Since the details of the characteristic bands of indolacetic acid are rapidly lost on enlargement, contact prints had to be used in this instance.

Spectroscopic differentiation of these two compounds is not possible, because of the similarity in the absorptions given by these two homologues.

2. *p*-Hydroxyphenylacetic acid (Fig. 8).—Extracts from a few normal as well as from pathological urines, chiefly after hydrolysis, gave the bands illustrated in Fig. 8a. These are in the same position as those given by *p*-hydroxyphenylacetic acid (8b).

No statement can be made at this time concerning the possible presence of *p*-hydroxybenzoic acid and *p*-hydroxyphenylpropionic acid. However, there was no spectroscopic suggestion for the presence of *p*-hydroxyphenyllactic acid and *p*-hydroxyphenylpyruvic acid in the urine investigated.

If indol-3-acetic and *p*-hydroxyphenylacetic acids are present in the same solution, both can be detected, as seen in Fig. 9. The narrow band in the region 2,920 Å., given by the indolacetic acid in the mixture of indolacetic and *p*-hydroxyphenylacetic acids, can also be seen in the original plate; it does not show plainly in the enlargement.

III. General Discussion of the Ether-Soluble Phenols of Urine

The presence of the following three groups of phenolic compounds in urine is well established:

Group 1. The monophenols,—phenol, *p*-cresol, and possibly *o*- and *m*-cresol (3, 4, 6-8, 13, 16, 19-21, 33, 36, 37, 41, 42, 69, 73, 81, 100, 103).

Group 2. The diphenols,—catechol, and possibly hydroquinone (3, 4, 8, 9, 15, 66, 74, 92).

Group 3. The estrogenic phenols,—estrone, estriol, and estradiol (23, 27, 29, 32, 60-62, 68).

The monophenols (group 1) can be easily steam-distilled, the diphenols (group 2) are very slightly steam-volatile, and the estrogenic phenols (group 3) are nonsteam-volatile. This difference makes possible the separation of group 1 from groups 2 and 3.

Those substances are present principally in their conjugated forms as glucuronides, ethereal sulfates, or both. Very little is known about the ratio of free to conjugated phenolic compounds in urine under either normal or pathological conditions. The conjugated compounds are either slightly soluble or insoluble in ether in contrast to the free substances which dissolve easily in that solvent. The conjugated phenol and *p*-cresol hydrolyze readily in the cold with mineral acids (68), whereas the conjugated estrogenic compounds split in the presence of mineral acids only after heating.

The isolation and identification by chemical methods of the very small amounts of phenolic compounds present in the urine in 24 hours is very difficult. For the identification of these substances color tests such as the Millon or the Folin-Denis reactions frequently are used, but they are not sufficiently specific to allow differentiation of the phenols (18, 27, 50, 64, 71, 72, 79, 93, 105). For clinical purposes the estrogenic phenols are determined either by biological methods or by the Kober reaction.





FIG. 10. (a)—Mixture of *p*-cresol and phenol. (b)—Steam-volatile phenolic compounds from unhydrolyzed urine (AE7).

FIG. 11. (a)—Mixture of *p*-cresol and phenol. (b)—*p*-Cresol. (c)—*o*-Cresol. (d)—*m*-Cresol.

FIG. 12. (a)—*p*-Cresol. (b)—Nonsteam-volatile phenolic compounds from hydrolyzed urine (late pregnancy) (AE6). (c)—Estrone. (d)—Nonsteam-volatile phenolic compounds from hydrolyzed urine (normal female) (AE6).

FIG. 13. (a)—Indigo blue. (b)—Absorption given by fraction AE6.

FIG. 14. (a)—Estriol. (b)—Catechol. (c)—Nonsteam-volatile phenols from unhydrolyzed urine (pernicious anemia) (AE6). (d)—Hydroquinone.

IV. Results of Spectroscopic Survey of the Ether-Soluble Phenols of Urine

*a. The fraction containing the total ether-soluble phenolic material (AE5 and BE5).—*The bands given by this fraction are in positions identical with those given by a mixture of *p*-cresol and phenol. The bands are very often decreased in their intensity by materials which give background absorption. The identification of the estrogenic phenols and diphenols in this fraction is not possible on account of the relatively high concentration of the *p*-cresol and phenol which give absorption bands in about the same region of the spectrum.

In most of these fractions obtained before, as well as after, hydrolysis, additional bands similar in position to those given by a solution of indigo blue were present in the region of 2,420 Å. and 2,480 Å. This compound was presumably produced from indoxyl by the fractionation procedure (18, 64, 72, 79, 105), and this will be discussed below.

*b. The steam-volatile phenolic compounds p-cresol and phenol (AE7 and BE7).—*The absorption bands of these fractions were the same as those described under a., except that the bands of indigo blue were not present.* So far only *p*-cresol and phenol have been detected spectroscopically in this fraction (Fig. 10). *p*-Cresol is present in larger amounts than phenol in the extract from both unhydrolyzed and hydrolyzed urines. In different specimens the relative concentrations of these two substances varied, and both were present in increased amounts after hydrolysis. No information is at hand to establish whether the *p*-cresol and phenol were present in free form in the urine before treatment, or were produced during the continuous ether extraction by acid hydrolysis of a conjugated compound at room temperature (68).

In Fig. 11 are shown the absorption spectra of the isomeric, ortho-, meta-, and para-cresols, and it is apparent that para-cresol can be differentiated from the ortho and meta compounds. It is reasonable to believe that if a great excess of phenol were not present, mixtures of phenol and para-cresol could be distinguished from mixtures containing phenol and meta-cresol, or phenol and ortho-cresol. It would be very difficult to distinguish between mixtures of phenol and ortho-cresol or phenol and meta-cresol.

c. The nonsteam-volatile phenolic compounds (AE6 and BE6).—I. The estrogenic phenols (Fig. 13). This fraction contained the phenolic female sex hormones and was of particular interest. The urines of normal adults of both sexes, as well as of twelve late pregnancies, were examined before and after hydrolysis. No absorption bands similar to those of the estrogenic phenols were given by extracts of 24-hour urine collections of normal individuals, or of unhydrolyzed urines of pregnant women. In fractions from the hydrolyzed urines of late pregnancy which contain relatively large amounts of estrogenic material, absorption bands in positions identical with those of estrogenic phenols were found (Fig. 12b). The same fractions were tested for biological activity in oophorectomized rats and were strongly estrogenic.

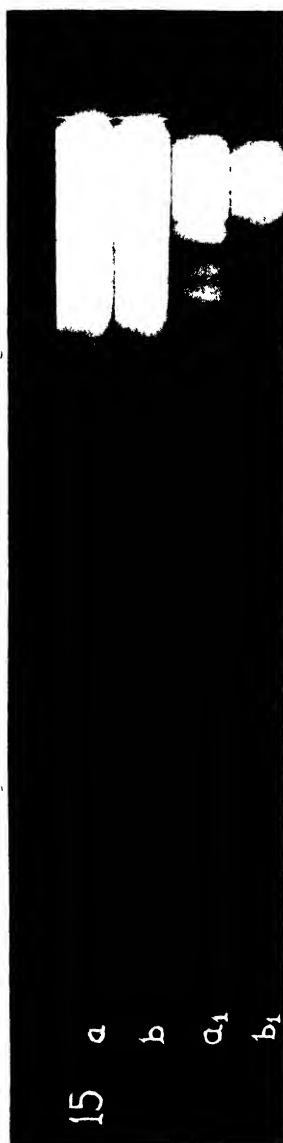


FIG. 15. (a)—Indigo red in low concentration to show the short wave length band. (a₁)—Indigo red in higher concentration to show the longer wave bands. (b)—Neutral compounds from unhydrolyzed urine in low concentration (AE4). (b₁)—Neutral compounds from unhydrolyzed urine in higher concentration (AE4)

FIG. 16. (A)—Phenol. (B)—*p*-Cresol. (C)—Mixture of phenol and *p*-cresol.

No absorption bands (Fig. 12d) and no positive biological reactions were obtained with similar fractions of urine from nonpregnant women.

A spectroscopic differentiation between estrone, estradiol, and estriol in this fraction is not possible without further chemical separation (23, 27-29, 32, 60-62, 68), since these substances give the same types of band structure in approximately the same positions (45).

2. Indigo blue (Fig. 13). When the fraction containing the estrogenic phenols was diluted, two bands in the region of 2,420 Å. and 2,480 Å. usually were found in positions similar to the bands given by indigo blue. An ethereal solution of indigo blue also gives a weak band at about 2,820 Å. which is usually too faint to be seen in the urine extract.

Indigo blue, as well as indigo red (18, 64, 72, 79, 105), is formed from its precursor, indoxyl, in the course of the treatment of the urine with mineral acids. The nonsteam-volatile indigo blue, only slightly soluble in ether, was partly extracted from the urine fraction.

3. Diphenols: catechol and hydroquinone (Fig. 14). Catechol and hydroquinone, if present in the urine, are found in the fraction containing the non-steam-volatile phenolic compounds. In one case of pernicious anemia in relapse the fraction from unhydrolyzed urine gave bands similar to those given by catechol (Fig. 14c). These bands are in the same general region as those given by the estrogens, but because the positions of the bands are different, these compounds can be easily differentiated, as seen in Fig. 14. Hydroquinone, if present, would absorb in a different region of the spectrum, the maximum being about 3,000 Å. (Fig. 14d).

For investigations concerning the presence of these diphenols in the urine, the fractionation procedure should be carried out in the presence of a reducing agent (sodium hydrosulfite) because the diphenols are easily oxidized and undergo rapid alteration in strongly alkaline solutions.

V. The Ether-Soluble, Neutral Compounds of Urine (Fig. 15).—General Discussion and Results

Among the neutral aromatic compounds giving absorption in the ultraviolet region of the spectrum, indigo red has been isolated from urines by a number of investigators (18, 64, 72, 79, 105). The ether extracts of unhydrolyzed urine regularly were of a bright reddish-purple color, but this was only rarely the case when the urine had been hydrolyzed before extraction. The intensity of the color varied in the extracts of urine from different individuals. When studied spectroscopically, this fraction gave a series of bands as seen in Fig. 15. Bands in similar positions were given by ethereal solutions of indigo red,⁴ a substance formed by the oxidation of indoxyl in alkaline urine (18, 64, 72, 79,

⁴ The indigo red was synthesized by Dr. Brooker and was obtained through the courtesy of Dr. Lloyd A. Jones, Eastman Kodak Company, Rochester, N. Y.

105). In acid urine a similar reaction is caused by mild oxidizing agents. As a result of the same process, indigo blue also is formed, but that compound is only slightly soluble in ether, whereas indigo red is more readily dissolved.

VI. Comparison of Results Obtained by Various Spectroscopic Technics

In Fig. 16, the spectra of an ethereal solution of pure phenol (A), *p*-cresol (B), and their mixture (C) are shown. A continuous light source was used. For comparison, the curves of these substances and their mixture obtained by means of a Spekker spectrophotometer are shown in Fig. 17. A spark was used as a source of light. In Fig. 17, D represents the absorption curve of a urinary fraction containing the steam-volatile phenols. The maxima and minima of this fraction are influenced by the background absorption of some material of unknown nature.

In Fig. 18, a comparison is made of the band spectra and their corresponding photometric tracing⁵ of the following materials: (A) the total ether-soluble phenols from urine; (B) the nonsteam-volatile fraction of A; (C) the steam-volatile phenols from A; and (D) a mixture of pure phenol and *p*-cresol.

DISCUSSION

The use of a continuous light source in the spectroscopic study of fractionated urinary extracts results in the appearance of well-defined bands, or groups of bands, which can be compared directly with those given by one reference compound or by a mixture of reference compounds. For the purpose of a qualitative survey, the spectroscopic method has been found rapid and satisfactory. This point is supported by a recent publication of Müller and Scholtan (65). Although the method is not a substitute for the standard quantitative methods of spectrophotometry, the qualitative procedure yields roughly quantitative information.

Since the fractions of urine are uniformly prepared, and since the spectroscopic work is done under standard conditions, the intensity of the bands from a fraction of urine can be compared with those given by reference solutions of known concentration, and thus a rough indication can be gained of the amount present. The application of standard quantitative spectroscopic methods to fractions of urine is hindered to some extent, however, by the presence of background absorbing material, which persists even after the preliminary chemical fractionation herein described.

The chemical methods of fractionation are the conventional procedures employed for the separation of acidic, phenolic, and neutral substances from organic solvents. A separation of the aromatic compounds was necessary in

⁵ We are indebted to Dr. E. G. Pickels of the International Health Division, Rockefeller Institute for Medical Research, for the photoelectric tracings of the plates.

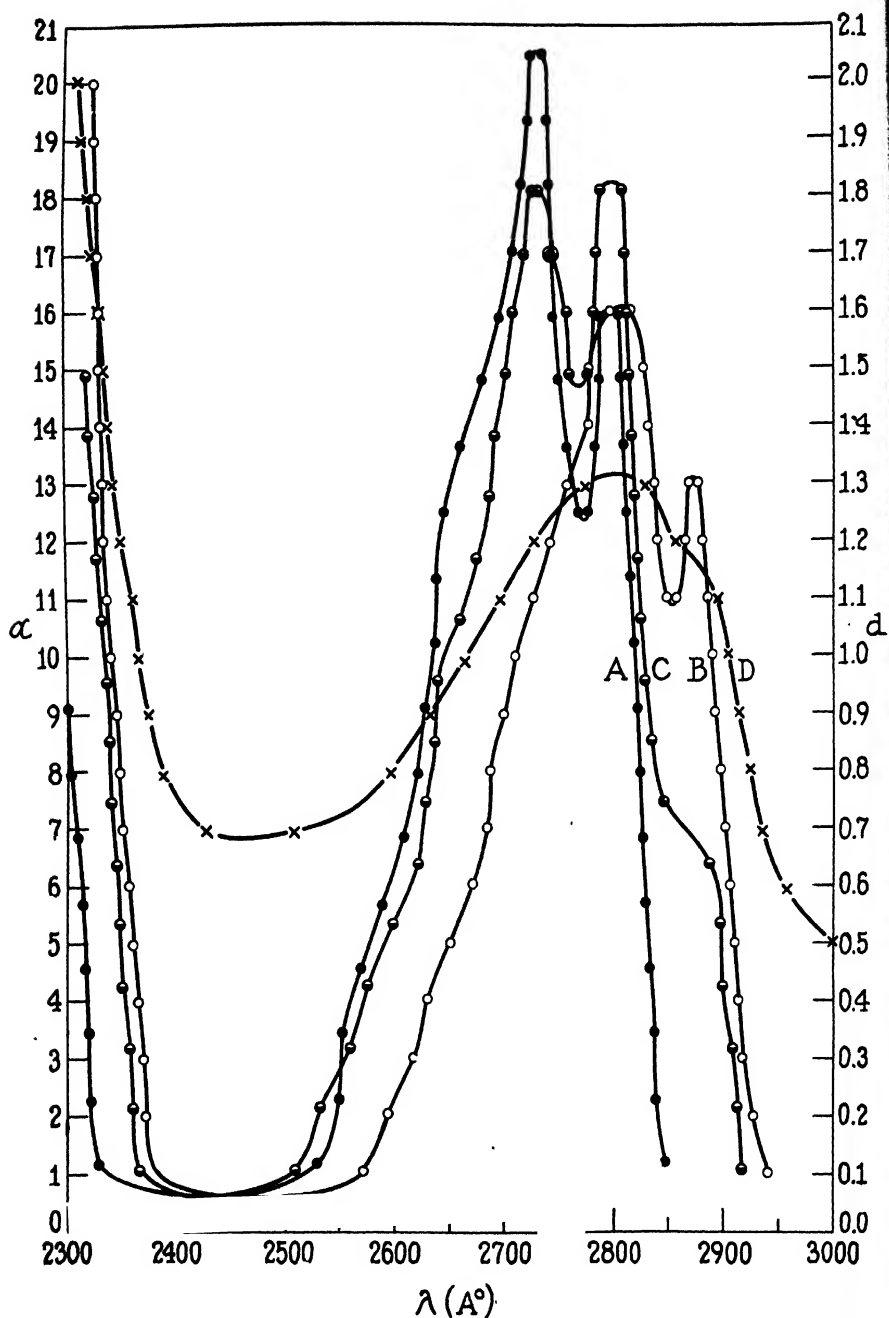


FIG. 17. Absorption curves of: (A)—Phenol. (B)—*p*-Cresol. (C)—Mixture of phenol and *p*-cresol. (D)—Steam-volatile phenols from hydrolyzed urine (BE7).

order to overcome the difficulties encountered in previous work in which it had been shown that spectroscopic examination of untreated materials did not show sufficient resolution for the detection of specific compounds. These disadvantages have been eliminated to a considerable extent by the present method of fractionation, but still further development of the chemical procedures is desirable. The aromatic bases and nonether-soluble aromatic compounds remain to be investigated.

The completeness of the chemical separation procedure may be inferred from the results of a similar fractionation of a mixture of pure aromatic acidic, phenolic, and neutral compounds which had previously been reported by other workers to be present in urines. From the presence of groups of bands in the proper chemical fractions and the absence of these bands in all of the other fractions, it may be inferred that the preliminary separation procedure was satisfactory. The same control was applied to all fractions obtained from urines, and on this basis, a second fractionation was necessary only occasionally. Within the limit of the sensitivity of the spectroscopic methods used here, all separations described in this paper were satisfactorily complete.

As shown in the results, the detection of various aromatic compounds by spectroscopic methods has been facilitated greatly by the use of fractionation procedures. However, the application of the method is limited for the following reasons: (a) Nonspecific, continuously absorbing material may blur or conceal the discrete absorption bands of substances present in low concentration. (b) The presence of compounds possessing absorption bands with high extinction coefficients will prevent the resolution of the bands of substances characterized by a low extinction coefficient. (c) The proximity of bands from two or three substances may cause overlapping to an appreciable extent, thus preventing identification. As an extreme case, overlapping can be complete, particularly in the case of such homologues as phenylacetic acid and phenylpropionic acid, both of which have been isolated from urines. These limitations can be partially overcome by more extended fractionation procedures. The differentiation of phenol from *p*-cresol in the same mixture is easily made spectroscopically. Similarly, hydroquinone and catechol, or benzoic acid and phenylacetic acid can be differentiated. Such a differentiation cannot ordinarily be accomplished by color reactions. On the other hand, estrone in a mixture of estrone, estriol, and estradiol can be detected by colorimetric methods. These three female sex hormones give practically identical absorption bands and cannot be distinguished spectroscopically in mixtures. From such data, it follows that spectroscopic and colorimetric methods should be used to supplement each other.

In the various fractions of human urine which have been studied, absorption bands were present which were given by compounds as yet unidentified. Further information concerning these absorbing substances will require their iso-

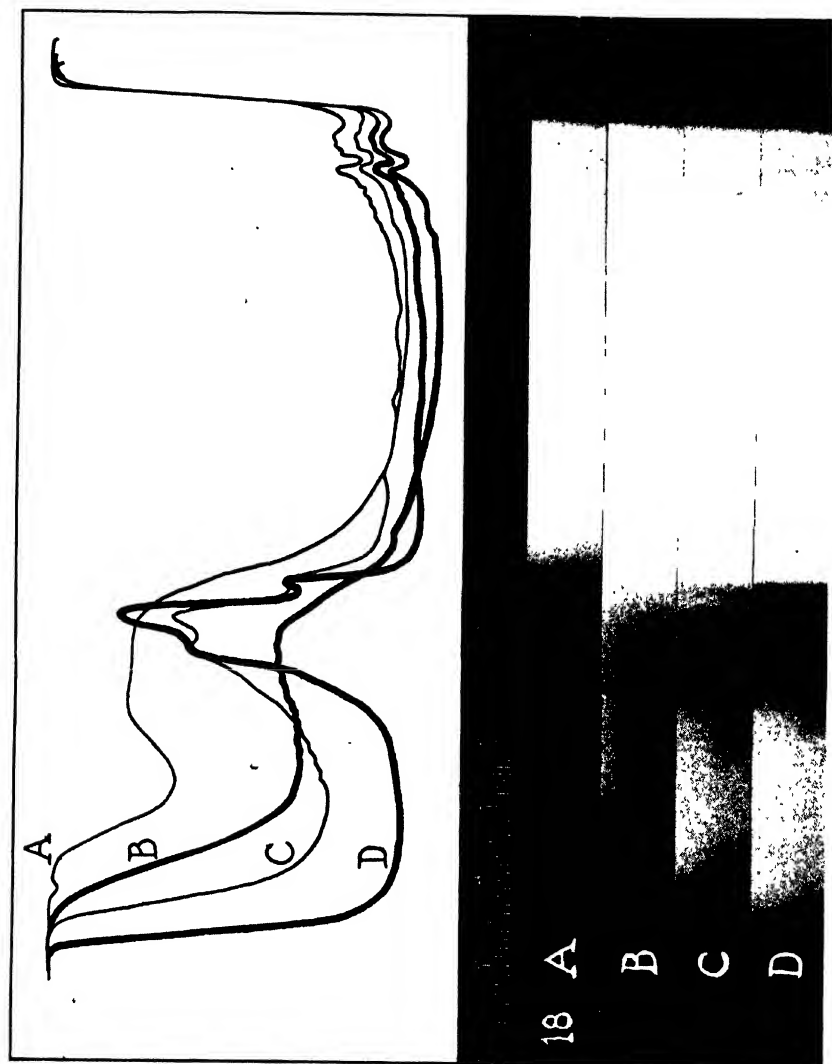


FIG. 18. Comparison of the band spectra and corresponding photometric tracings. (A)—Ether-soluble phenols from hydrolyzed urine (BE5). (B)—Nonsteam-volatile fraction from (A). (C)—Steam-volatile fraction from (A). (D)—Mixture of pure phenol and *p*-cresol (1 part phenol and 9 parts *p*-cresol).

lation in sufficient quantities to allow chemical study. For their isolation, however, spectroscopic methods will be helpful in following the chemical procedures. On the basis of the findings herein described, it appears that the spectroscopic method is of great value in following the metabolism of certain aromatic substances not normally present in excreta.

SUMMARY AND CONCLUSIONS

A method for the treatment and preliminary separation of the ether-soluble substances normally present in urine into groups suitable for the study of absorption spectra is described. Following such a separation, absorption spectra do not present the confused picture of overlapping bands which has been found in the past with whole urine. While no claim is made for complete identification of any one substance, its probable nature may be inferred from comparison with the absorption spectra of certain aromatic substances which are known from previous chemical study to be present in normal urine.

If these pure aromatic substances are mixed and subjected to the fractionation procedures, the resulting absorption bands in the different fractions are in similar positions to the absorption bands from the same fraction obtained from the urine.

Thus far, we have had occasion to study some 150 different urines from 48 normal and diseased individuals, and have not encountered appreciable variations from the findings presented. Possibly the same sort of separation and classification will enable a clearer picture of the absorption spectra of other body fluids to be made.

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THE SPECTROSCOPIC STUDY OF BIOLOGICAL EXTRACTS

II. THE DETECTION, ISOLATION, AND BIOLOGICAL EFFECTS OF THE METABOLITES OF 1,2,5,6-DIBENZANTHRACENE*

BY K. DOBRINER, M.D., C. P. RHOADS, M.D., AND G. I. LAVIN, Ph.D.

(From the Memorial Hospital for the Treatment of Cancer and Allied Diseases and the Hospital of the Rockefeller Institute for Medical Research)

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The application of chemical fractionation and qualitative spectroscopic methods to the detection of ether-soluble aromatic compounds from human urine has been reported in a previous publication (14). The results of the spectroscopic survey demonstrated that similar fractions obtained from many individual human urines showed a remarkable uniformity of absorption. A study of the absorptions given by the urine of various animal species (15) indicated the presence of certain differences as compared with the absorptions given by similar fractions from human urines. The bands given by the urine of various individuals of a single species were remarkably uniform. Because of this fact, the occurrence of new bands suggests the presence of compounds not normally present. Therefore, the qualitative spectroscopic method may be employed to follow the metabolism of various compounds which absorb in the ultraviolet region of the spectrum. Many hydrocarbons which possess carcinogenic activity absorb in this region. The fate of anthracene, naphthalene, methylcholanthrene (15), and dibenzanthracene (12) has been investigated by the described combination of chemical fractionation and qualitative spectroscopic methods. This publication is concerned with the metabolic fate of 1,2,5,6-dibenzanthracene¹ in several different animal species.

Material

Mice, rats, rabbits, dogs, and monkeys were employed. The animals were maintained on normal laboratory diets of constant composition and were kept in standard metabolic cages which were cleaned with alcohol and ether after each collection of the excreta. The urine and feces were collected in 3- or 4-day periods. Adequate control periods were allowed before injection. 1,2,5,6-Dibenzanthracene (DBA) was injected subcutaneously employing a solution of 500 mgm. of the white, colorless compound (Eastman) dissolved in

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¹ Henceforth, "DBA" will be used in place of "dibenzanthracene" except in special instances.

75 cc. of olive oil. Animals of the various species received the following doses of DBA once or twice weekly: mice, 10 to 20 mgm.; rats, 20 to 30 mgm.; rabbits, 50 mgm.; monkeys, 200 mgm.; dogs, 500 mgm. For the spectroscopic survey, the excreta were worked up at the end of each 3-day period. For the isolation procedures, the excreta from several collection periods were pooled and were kept in the refrigerator.

METHODS

I. Chemical

The methods for the extraction and fractionation of the ether-soluble compounds from the excreta and tissues were, with some minor modifications, the same as those described in a previous publication (14).

a. *Urine and bile*.—The ether-soluble fractions from urine and bile were prepared by a continuous ether extraction for 72 hours in a Kutcher-Steucl apparatus. Four primary ether extracts were obtained from the same urine or bile treated successively as follows (Fig. 1): 1. First the material was acidified to pH 1 with concentrated hydrochloric acid (ether extract AE1). 2. It was then made alkaline to pH 8 with solid sodium bicarbonate (ether extract BE1). 3. It was reacidified to pH 1 with concentrated hydrochloric acid and refluxed on a hot plate for one-half hour or autoclaved for two hours at 115°C. at 15 pounds (ether extract CE1). 4. The hydrolyzed material was made alkaline with solid sodium bicarbonate to pH 8 (ether extract DE1). The fractions containing the acidic, phenolic, and neutral substances were prepared from the primary ether extracts obtained from both nonhydrolyzed (AE1) and hydrolyzed urine or bile (CE1). The primary ether extracts, BE1 and DE1, contained the basic compounds obtained from the nonhydrolyzed and hydrolyzed urines respectively. The ethereal subfractions were brought to a convenient volume (10 to 50 cc.) on the steam bath and their absorption spectra in the ultraviolet region were taken.

b. *Feces and intestinal tract content*.—The feces were acidified with concentrated hydrochloric acid, mixed with anhydrous sodium sulfate, ground to a fine powder, and mixed with sand. They were then extracted with ether for 72 hours in a Soxhlet apparatus. The ether extracts were fractionated as described for extracts of urine (see section a.), brought to a volume of 20 to 100 cc., and examined spectroscopically.

c. *Tissues*.—The tissues were ground up in a mortar and extracted thoroughly with a mixture of alcohol and ether. The extracts were fractionated as described under a.

d. *Whole rats*.—Carcasses of DBA-injected rats were ground up and saponified with 10 per cent alcoholic potassium hydroxide. After acidification with hydrochloric acid, the material was extracted for 72 hours with ether. Fractionation of the ether extract was carried out as described under a.

II. Spectroscopic

The spectroscopic methods used were described in a previous publication (14).

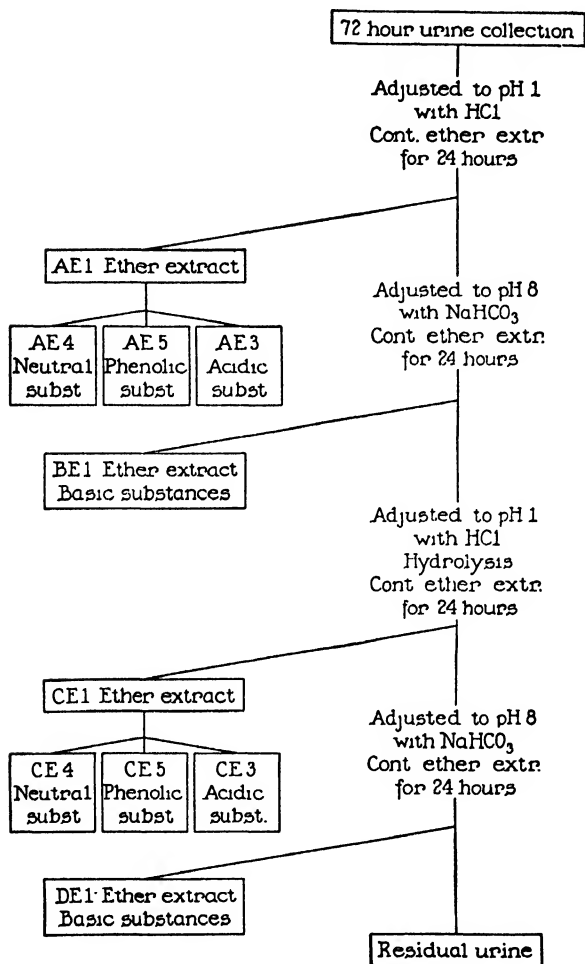


FIG. 1. Scheme for the separation of the ether-soluble substances from urine, feces, and tissues.

RESULTS

I. Spectroscopic Survey of the Crude, Neutral, Phenolic, Acidic, and Basic Fractions

For this survey, the absorption spectra of similar fractions obtained from the excreta and tissues of animals before and after the injection of DBA were

compared. By this procedure, additional absorption bands which were present after the injection of DBA could be detected. The bands present in the fractions of the material from normal animals will not be discussed in detail if not relevant to the detection of new absorption bands which could be attributed to the presence of DBA or its derivatives. The results obtained are summarized in Table I.

A. *Fraction containing the neutral, ether-soluble compounds.* 1. *Urine.*—In this fraction (AE4) of the urine from some uninjected animals, particularly monkeys, bands in positions identical with those given by indigo red were observed. After the injection of DBA, this fraction from the urine of rabbits and monkeys occasionally gave a series of sharp bands which were not present in the similar fractions from the urine of normal animals. Similar bands were usually present in the neutral fraction from the urines of DBA-injected rats and mice. After the hydrolysis of the urines, these bands could not be found. The positions of the bands were identical² with those given by an ethereal solution of DBA. Those given by the urinary fractions were of comparatively low intensity and were obscured and diminished by a relatively large amount of background absorption. The spectroscopic findings suggested the presence of DBA in the urine of the animals injected with that compound.

The possibility of a leakage after injection of the olive oil containing DBA cannot be excluded. Furthermore, the urine may wash down fecal particles. The spectroscopic suggestion of the presence of DBA in the urines of injected rabbits and monkeys may result from this artifact, because the bands are not found regularly. In the case of injected rats and mice, on the other hand, the regular occurrence of these bands indicates that these species may excrete a small amount of DBA in the urine.

2. *Feces.*—The neutral fraction (AE4) from the feces of normal animals was highly pigmented and contained a relatively large amount of background absorption. Occasionally diffuse absorption bands of relatively low intensity were present. In the fraction obtained from the feces of mice and rats after DBA injection, bands in positions identical with those given by DBA were regularly present. Their intensity in the region of 3,100 to 4,500 Å. was often considerably decreased due to background absorption. In the fraction obtained from feces of injected rabbits, dogs, and monkeys, bands of low intensity in positions identical with those of DBA were observed occasionally, usually in the first collection after injection. In the neutral fecal fraction from certain uninjected monkeys, a series of diffuse bands between 3,650 Å. and 3,900 Å. and four well-defined bands in the region of 3,000 Å. to 3,500 Å. are occasionally present, but if present, the bands of DBA could readily be detected and distinguished.

² Hereafter the term "identical" as applied to absorption bands will be used to designate bands in identical positions.

The excretion of DBA in the feces of rats and mice is suggested by the regular finding of absorption bands in positions identical with those of DBA. In rabbits, dogs, and monkeys, however, this finding appears to be due to the leakage of oil containing DBA.

3. *Intestinal contents*.—It was uncertain whether the DBA detected spectroscopically in the fractions from the urine and feces of certain species represented an excretory product or whether its presence in these fractions was an artifact. Therefore, experiments were undertaken to elucidate this point.

In the neutral fraction obtained from the intestinal contents of mice injected with DBA, bands of very low intensity in positions identical with DBA were present. The similar fraction from the intestinal contents of rats showed the

TABLE I
Summary of the Excretion and Conversion of 1,2,5,6-Dibenzanthracene (DBA) and Dihydroxydibenzanthracene in Different Species

Animals	DBA Neutral fraction					(OH) ₂ -DBA Phenolic fraction				
	Urine	Feces	Intes- tinal tract contents	Liver	Bile	Urine	Feces	Intes- tinal tract contents	Liver	Bile
Mice.	+	+	(+)	0	..	+	+	(+)?	0	
Rats	+	+	+	(+)	.	+	+	+	0	
Rabbits	(+)?	(+)	(+)	(+)	(+)	+	+	+	0	(+)
Dogs.	(+)?	(+)?	.	.	.	(+)	(+)	
Monkeys.	+	0	.	0		

+ Positive; (+) traces; (+)? questionable; 0 negative; . not studied.

same bands in greater intensity. In the same fraction of rabbits, the bands of DBA were present in low intensity.

From this experiment it appears that the presence of DBA in the feces of mice, rats, and rabbits is not due to an artifact, but is the result of the excretion of DBA into the intestinal tract following parenteral injection.

4. *Bile*.—Only a few cubic centimeters of bile could be obtained from rabbits injected with DBA. After fractionation of the material, the neutral fraction showed only faint bands suggesting the presence of the hydrocarbon.

5. *Livers*.—The neutral fractions of mouse, rat, and rabbit livers of animals injected with DBA showed spectroscopically a number of bands different from those of DBA. A band at 2,990 Å., seen in the neutral fraction obtained from the rats and rabbits, might indicate the presence of a trace of DBA. In the same fraction obtained from mouse livers, there was no suggestion of a band in this position.

6. *Whole rats*.—The neutral fraction from rats injected with DBA showed intense bands identical with those of DBA.

B. Fractions containing the phenolic compounds. 1. *Urine and feces of rats and mice.*—In this fraction obtained from the urine of uninjected animals (AE5), diffuse absorption bands were found in the region of 2,600 Å. and 2,900 Å. In similar fractions obtained from the urines after DBA injection, groups of bands were observed which were not present in this fraction from the urine of normal animals. The bands were in the following positions: a strong band at 2,880 Å., 3 bands of low intensity between 3,200 Å. and 3,500 Å., and 2 sharp bands between 3,800 Å. and 4,100 Å. The absorption bands in the fractions from mice and from rat urines were identical. The spacing of the bands was similar to that of DBA, but a comparison of the exact positions of the bands with those of DBA shows that the former are shifted by about 50 Å. towards the higher wave lengths. The absorption spectra given by similar fractions of hydrolyzed urine of normal and DBA-injected animals showed no bands suggesting the presence of DBA metabolites.

In the phenolic fraction obtained from the feces of normal animals, a material was present which gave a diffuse absorption from 2,700 Å. to 2,900 Å. and from 3,000 Å. to 2,500 Å. The intensity of the bands varied in different preparations. In the fractions of the feces of the DBA-injected animals, bands were observed in positions identical to those described for the urine of the injected animals.

In the phenolic fraction of the urine and feces from rats and mice injected with DBA, a series of bands was observed which are similar in grouping to, but in different positions from, the bands characteristic of DBA, a fact which indicated the presence of a phenolic derivative of DBA. The same metabolite may be produced by rats and mice, since the same bands are observed in the phenolic fractions from these two species.

2. *Urine and feces of rabbits.*—In the phenolic fraction of the urines of uninjected rabbits, diffuse absorption bands were present somewhat similar to those found in the same fraction of the urine from mice and rats. Of a series of sharp bands given by the fraction obtained from the urines of animals injected with DBA, two were relatively intense in the region of 2,850 Å. to 3,050 Å.; three bands of lower intensity were present in the region of 3,800 Å. to 4,100 Å. A comparison of the absorptions given by the fraction obtained from the urines after hydrolysis showed no difference between the excreta of normal and of DBA-injected animals.

A varying amount of background absorption was given by the phenolic fractions of the feces obtained from normal rabbits. In the similar extract of the feces of animals injected with DBA, bands were present in positions identical with those given by the same urinary fraction.

The bands observed in the phenolic fraction from the urine and feces of injected rabbits showed groupings somewhat similar to those of DBA but slightly shifted towards the longer wave lengths. A comparison of the positions

of the absorption bands given by the phenolic fraction of the excreta of injected rabbits with the absorption bands given by the DBA derivatives excreted by injected rats and mice showed that the shift of the bands of the rabbit compound in the region of 3,000 A. was less than that of the compound excreted by rats and mice; in the region of 3,100 A. to 3,500 A., the shift of the bands of the rabbit compound was greater, and the two bands in the region 3,800 A. to 4,110 A. were in identical positions.

The presence of bands in the phenolic fraction of urine and feces of injected rabbits in positions similar to, but not identical with, those of DBA suggests the presence of a phenolic derivative of DBA. The comparison of the positions of the bands given by the phenolic fraction of rabbits' excreta with those given by a similar fraction of the excreta of rats and mice shows that the positions are not identical. This difference of position indicates that the phenolic derivative of DBA excreted by rabbits is a different compound from that excreted by mice and rats. The lack of similar bands in the urines after hydrolysis indicates that the phenolic compound is not freed by the hydrolysis of an ether-soluble ethereal sulfate or glucuronide.

3. *Urine and feces of monkeys and dogs.*³—The phenolic fractions of urines and feces of monkeys both before and after hydrolysis and before and after the injection of DBA showed no differences in ultraviolet absorptions; no bands were observed which were suggestive of a phenolic derivative of DBA in these two species.

The phenolic fractions of the urines and feces of dogs injected with DBA showed faint absorption bands in the region of 2,900 A. to 3,100 A. which were not present in the same fractions obtained from the excreta of uninjected dogs.

4. *Intestinal tract contents.*—The phenolic fraction obtained from the intestinal contents of mice injected with DBA showed no bands characteristic of a DBA derivative. In the phenolic fraction of rats injected with DBA, bands of a relatively high intensity were present and the positions of these bands were identical with those of the excreted phenolic conversion product found in rat urine and feces. The phenolic fraction from DBA-injected rabbits showed a series of bands of high intensity whose positions corresponded with those of the conversion product present in the urine and feces of rabbits.

5. *Bile.*—In the phenolic fraction from rabbit bile, a number of bands different in position from the phenolic conversion product excreted in urine and feces were observed. One sample, however, showed bands in positions identical with those of the excreted phenolic conversion product.

6. *Livers.*—In the phenolic fractions obtained from the livers of mice, rats,

³ Since this manuscript was submitted, the phenolic fraction of the urine of dogs injected with DBA was purified further. A phenolic DBA derivative is present which differs spectroscopically both from the phenolic derivatives excreted by rats and mice, and by rabbits.

and rabbits injected with DBA, no bands were observed which could suggest the presence of a phenolic conversion product of DBA.

7. *Whole rats*.—In the phenolic fraction obtained from whole rats injected with DBA, a large amount of generally absorbing material obscured faint bands in positions identical with the phenolic conversion product of DBA found in the urine and feces of injected rats.

C. *Fractions containing the acidic compounds, AE3 and BE3*.—A comparison of the bands given by this fraction obtained from the urine and feces of animals of all species investigated before and after the injection of DBA showed no bands which suggested the presence of a DBA derivative.

D. *Fractions containing the basic compounds*.—The basic fraction of urines of mice, rats, rabbits, and dogs gave no bands which suggested a basic derivative of DBA. The similar fraction from the urine of injected monkeys showed irregularly a series of bands which were not present in the basic fraction of normal monkeys. Studies on the nature of this absorbing material are in progress.

E. *Investigation of the presence of ether-insoluble DBA derivatives*.—The presence of ether-insoluble derivatives of DBA was investigated in the urines of different species after hydrolysis and autoclaving of the urine. By this treatment ether-soluble DBA or DBA derivatives may be split off from ether-insoluble conjugated DBA compounds. In the phenolic fraction from rabbit urines, bands of low intensity were only very rarely observed in positions identical with the bands of the phenolic derivative obtained from unhydrolyzed urine. The fractions obtained from the urines of other species showed no bands which indicated the presence of DBA or a DBA derivative.

The unhydrolyzed urines of rabbits and rats, after the preliminary ether extraction, were extracted with butyl alcohol or chloroform solvents which may dissolve conjugated DBA derivatives. After these fractions had been taken up in alcohol and examined spectroscopically, no suggestion of a DBA derivative was found.

In the course of the fractionation procedure DBA derivatives might be rendered less ether-soluble. Therefore, precipitates which formed during the fractionation procedure were dissolved in alcohol and absorption spectra were taken. However, no suggestion of a DBA derivative was found.

II. The Purification and Isolation of the Phenolic Derivatives of DBA from the Excreta of Mice, Rats, and Rabbits (12)

The phenolic derivatives of DBA excreted by mice and by rats were isolated from the combined urine and feces of each species. In rabbits, the phenolic derivative was isolated both from the urine and feces. The excreta were collected from 50 mice, 30 rats, and 16 rabbits. The urine and feces were kept in the icebox and worked up in pooled collections of the material excreted dur-

ing 10 days. The ether-soluble phenolic fractions were obtained as described under METHODS, and the fractions from each 10-day period were combined for the final purification procedures. With minor variations, the same technic was used for the excreta of all the species employed. All purification procedures were controlled by fluorescence and spectroscopic methods.

A. *Primary purification of the phenolic fraction.*—The combined ethereal solutions containing the phenolic compounds were extracted with 10 per cent sodium carbonate until no further pigments could be removed. The ethereal solution was then extracted several times with 10 per cent sodium hydroxide. The combined sodium hydroxide solutions were acidified with hydrochloric acid and extracted three times with ether. The procedure was repeated several times. By this means, relatively large amounts of impurities were eliminated with a minimal loss of the phenolic compounds as judged by the spectroscopic method. The ether was evaporated and the oily residue was dried in a vacuum desiccator and submitted to high vacuum distillation as described below.

B. *High vacuum distillation.*—High vacuum distillation was carried out on a Hickman oil diffusion pump at a pressure between 10^{-4} and 10^{-5} mm. of mercury. Preliminary experiments showed that fractions collected between the following temperatures were satisfactory: 25–50°C., 50–90°C., 90–150°C., 150–250°C. In the fraction collected between 25–50°C., a considerable amount of oily material, phenol, and *p*-cresol were present. This fraction was discarded since it gave no bands spectroscopically which indicated the presence of a DBA derivative. In the fraction collected between 90 and 150°C., only small amounts of DBA derivatives were detected spectroscopically. In the fraction collected between 150 and 250°C., most of the derivative was present. Although no quantitative studies have been made, it appears that at high temperatures some destruction of the DBA derivatives takes place.

C. *Chromatographic adsorption analysis.*—Since the fractional crystallization of the distillates collected between 90 and 150°C. and 150 and 250°C. was not successful, except for the material from the urinary fraction of injected rabbits, further purification was carried out by chromatographic adsorption analysis. Preliminary studies controlled by spectroscopic and fluorescence procedures showed that the following technic was satisfactory: Aluminum oxide Brockmann (Merck) was used as the adsorbent. The bore of the adsorption columns varied from 10 to 20 mm. in diameter. The amount of aluminum oxide used was roughly 50 times the weight of the material to be purified. The height of the adsorption column was 20 to 30 times that of the diameter of the glass column used. The solvents were forced through the column by air pressure supplied by water flowing from a height of 5 feet into a closed container. A calcium chloride tube was inserted between the closed container and the column to absorb the water vapors. The columns were prepared in a mixture of benzol

and ether (1:1), and the following solvents were used successively in 100 cc. portions: ether-benzol (1:1), ether, ether-acetone (9:1), ether-acetone (1:1), acetone, acetone-methyl alcohol (9:1), acetone-methyl alcohol (1:1), methyl alcohol, ethyl alcohol, and ethyl alcohol-water (1:1). Since DBA and DBA derivatives give strong fluorescence, the quantity of a solvent necessary for the elution of the fluorescent materials from the column was controlled by means of a mercury lamp with a Wood filter. The rabbit and rat derivatives were eluted in mixtures of acetone and methyl alcohol. The use of the complete sequence of solvents described is of value in that a large amount of oil and pigments may be removed before the elution of the desired material.

TABLE II
Chromatographic Analysis of Crude Dihydroxydibenzanthracene Preparations

Solvent cc.*		Rabbits		Rats	
		Fluorescence	Spectroscopic	Fluorescence	Spectroscopic
EB 1:1	50	Blue	Pale blue	. .
EB 1:1	50	Blue	Blue	.
E	50	Pale blue	...	Violet	.
E	50	Light blue	
10% AcE	50	Blue +	
50% AcE	50	Blue +	. .
Ac	50	Light green	Phenol <i>p</i> -cresol	Blue +	
Ac	50	Blue +++	(+)	Blue +	.
10% MA Ac	50	Blue +++	++	Blue +	.
10% MA Ac	50	Blue +++	+++	Blue +	
10% MA Ac	50	Blue +++	+++	Blue +(+)	(+)
50% MA Ac	50	Blue +++	+++	Blue +++	+++
50% MA Ac	50	Blue +++	++	Blue +++	+++
MA	50	Blue ++	+	Blue +++	+
EA	50	Blue +	(+)	Blue +	(+)

* E = ether; B = benzol; Ac = acetone; MA = methyl alcohol; EA = ethyl alcohol.

Because of the differences in the impurities which influence the speed of elution in each preparation, the exact point in the sequence of solvents at which the DBA derivative will be eluted cannot always be predicted. The different eluates were evaporated *in vacuo*, dissolved in measured amounts of ether, and investigated spectroscopically. The fractions containing the DBA derivative were combined and the chromatographic procedures were repeated two or more times, according to the amount of impurities present. The compound excreted by rabbits is eluted from the column earlier in the sequence of solvents than is the rat compound. This fact is in agreement with the higher solubility in ether of the pure compound produced by rabbits than of the rat compound. The details of a typical chromatographic adsorption analysis are shown in Table II.

*D. Isolation and properties of the phenolic derivative.*⁴—Preparations from the same species which gave fluorescence and strong ultraviolet absorption were combined, purified in alcoholic solution with a very small amount of Norite, and crystallized from mixtures of acetone and petroleum ether. The melting points of the different preparations are given in Table III. The material isolated from the urine of rabbits melted at 355–358°C. The elementary analyses were: Calcd. for $C_{22}H_{14}O_2$: C, 85.14; H, 4.54. Found: C, 85.04; H, 5.07.

The mixed melting point of equal amounts of the rabbit compound with the rat compound gave a melting point of 335–339°C., a depression of 20°C. A melting point in a closed tube of the rat compound as determined by Fieser and Cason was 390–400°C. corr. (7). They reported that this preparation did not give any depression of the mixed melting point with the synthetic

TABLE III
Melting Points of Natural and Synthetic Dihydroxydibenzanthracenes

Animals	Source	Dobriner <i>et al.</i>	Cason and Fieser (7)	Cason and Fieser (7)	Boyland and Levi (5)
Rabbits . . .	Urine	355–358°C.	..		340–350°C.
Rabbits. . .	Feces . .	356°C.—a†	..		
Rats	Urine and feces	Above 370°C.—b‡	390–400°C.*	415–418°C.†	
Mice	Urine and feces	Above 370°C.			..

* Melting point of the natural material isolated by Dobriner *et al.*

† Melting point of synthetic 4',8'-dihydroxydibenzanthracene.

‡ Mixed melting point of a and b, 335–339°C.

4',8'-dihydroxydibenzanthracene. The synthetic compound of Fieser and Cason (7) melted at 415–418°C. corr.

III. Spectroscopic Investigation of the Purified Compounds

The absorption spectra of DBA and the phenolic derivatives of rabbits, rats, and mice are shown in Fig. 2. The absorption curves of the phenolic derivatives from rats and mice are identical. The spectra were measured by means of a small Hilger spectrograph, a Spekker photometer, and a metallic spark as a light source. All the substances measured were dissolved in absolute ethyl alcohol.

In Fig. 3 are shown the absorption bands of the same compounds as measured by the Hilger spectrograph with a hydrogen discharge tube as a source of

⁴ The authors wish to thank Dr. L. C. Craig of the Rockefeller Institute for Medical Research for the purification and recrystallization of the isolated dihydroxydibenzanthracenes.

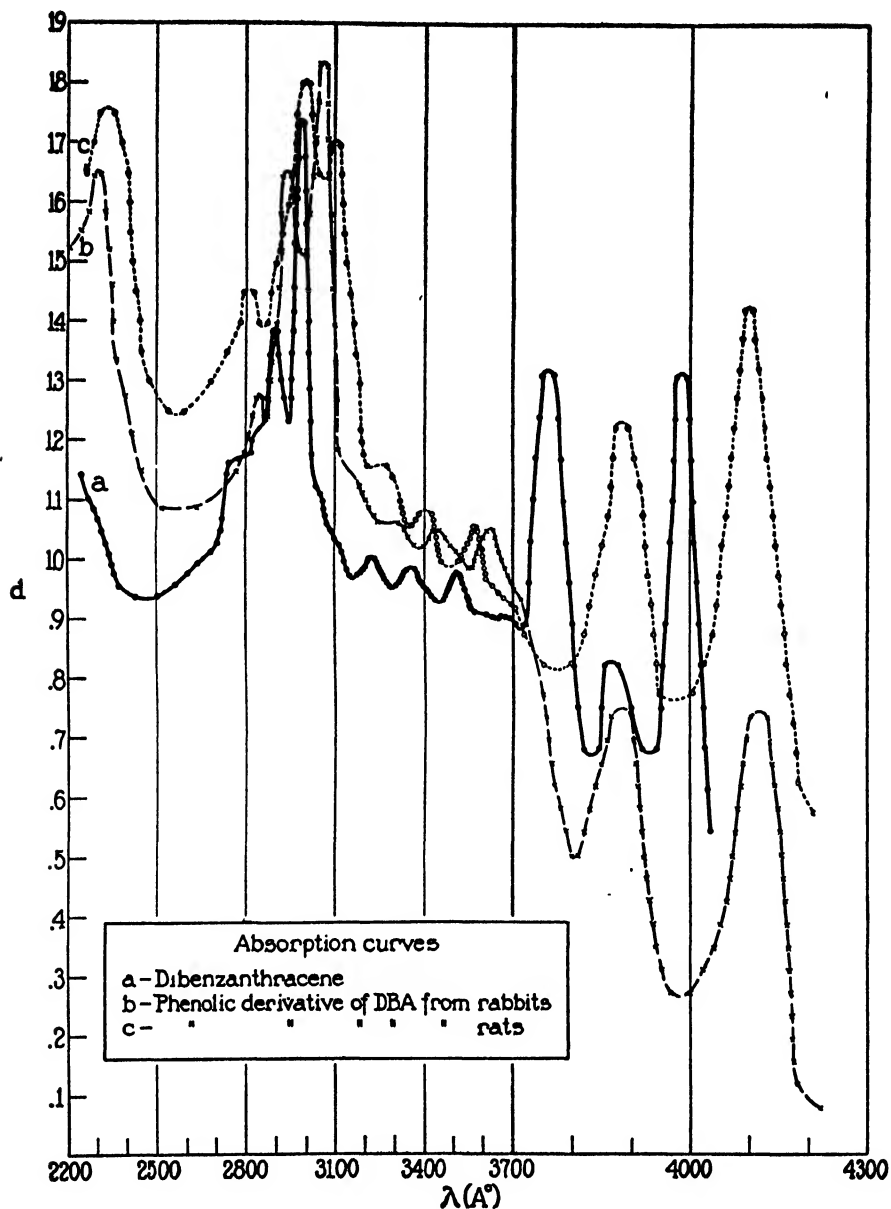


FIG. 2

light. The two lower spectra (II and III) are those given by dilutions of the solutions from which the top spectra (I) were obtained. Those marked "A" represent the bands given by DBA, those marked "E" represent the ones

given by the phenolic derivative from rabbits, "C" indicates the bands given by the phenolic derivative of rats, and "D" those from mice.

In the same figure the positions of the phenolic derivative from rats and mice are compared with the bands (B) given by the synthetic 4',8'-dihydroxydibenzanthracene obtained through the courtesy of Dr. Fieser. The absorption spectra were taken in alcoholic solutions. The positions of the bands from the natural DBA derivative excreted by mice and rats and the synthetic product are identical.

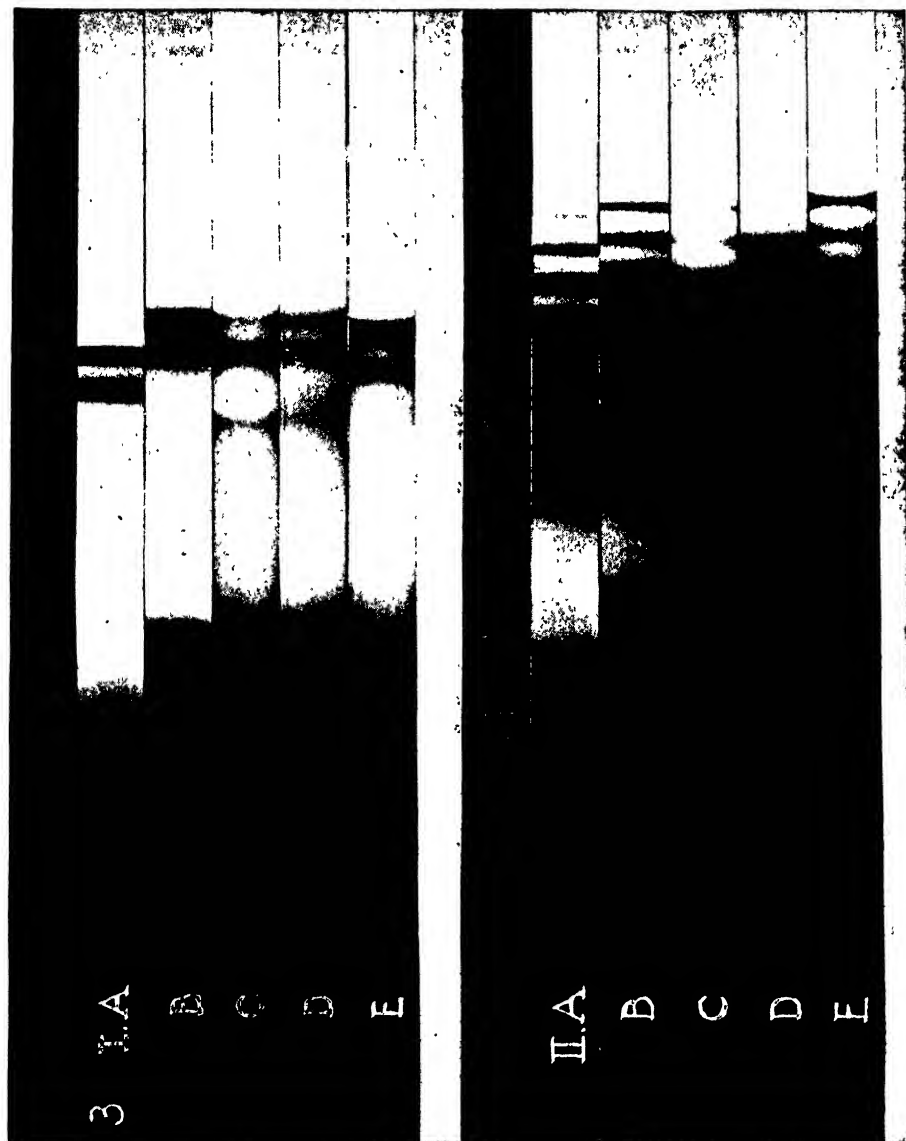
In Fig. 4 the absorption curves given by the phenolic derivative of rabbits isolated in this laboratory (b) and by the phenolic derivative of DBA isolated from rabbit urine by Boyland and Levi (5) (a) are depicted. The shapes of the curves are identical.

IV. Tests for Carcinogenic Activity of the Rabbit Compound

Twenty-two mice of a pure strain of known susceptibility to DBA were obtained through the courtesy of Dr. Clara Lynch of the Rockefeller Institute for Medical Research. Eleven animals were employed to test the carcinogenicity of the phenolic derivative of DBA. Each animal was injected with 1 mgm. on the following dates: 11/28/39 and 12/2/39, and with 2 mgm. on 1/4/40 of DBA or the phenolic derivative of DBA, respectively. The solution injected contained 4 mgm. of DBA, or the phenolic derivative, per cc. of lard. Two of the animals injected with the phenolic derivative died after 3 weeks without evidence of a tumor. The 11 mice injected with DBA and 9 remaining mice injected with the phenolic derivative were killed 240 days following the first injection. All the DBA-injected animals showed at autopsy a sarcoma at the site of injection, and none of the 9 mice injected with the phenolic derivative of DBA developed tumors.

V. Fate of the Dihydroxydibenzanthracene Excreted by Rabbits after Injection in Mice

The urine and feces of the mice injected with the phenolic derivative of DBA excreted by rabbits were collected for 6 days following the first injection and fractionated as described under METHODS. The absorption spectra of the neutral and phenolic fractions were investigated. In the neutral fractions of the urine and feces, no bands were seen which were characteristic of DBA or of a DBA derivative. In the phenolic fractions from urine and feces, respectively, bands were present in positions identical with the bands given by the phenolic derivative excreted by rabbits. In the urine, only traces of this material were present, while in the feces, a relatively high concentration was observed, as judged by the results of the spectroscopic method.



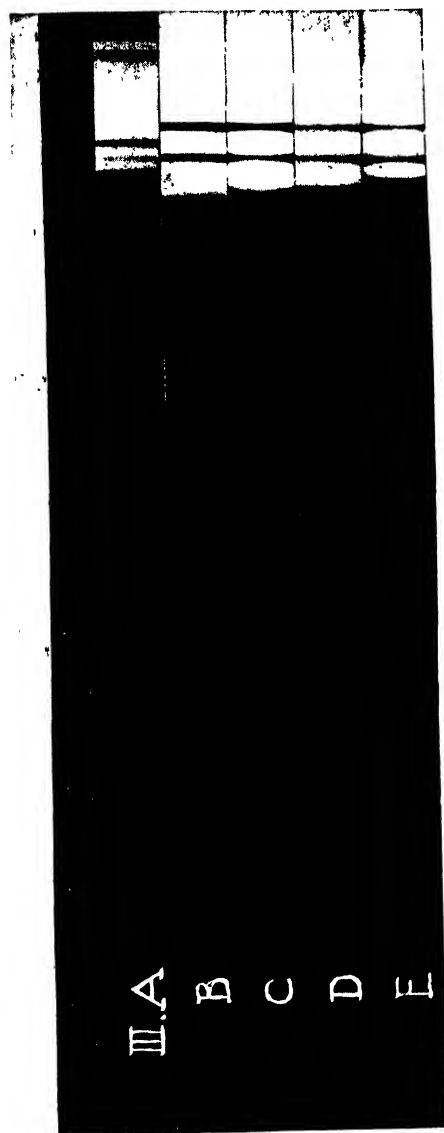


FIG. 3. Comparison of the absorption bands given by: (A)—1,2,5,6-Dibenzanthracene. (B)—4',8'-Dihydroxydibenzanthracene synthesized by Fieser and Cason. (C)—Dihydroxydibenzanthracene excreted by rats after the injection of 1,2,5,6-dibenanthracene. (D)—Dihydroxydibenzanthracene excreted by mice after the injection of 1,2,5,6-dibenanthracene. (E)—Dihydroxydibenzanthracene excreted by rabbits after the injection of 1,2,5,6-dibenanthracene

TABLE IV
Excretion and Conversion of 1,2,5,6-Dibenzanthracene (DBA) in Groups of Animals of Different Species

Animals*	DBA mgm				Dihydroxy DBA			
	Urine	Feces	Intestine	Total	Urine	Feces	Intestine	Total
20 Mice	a				0.08	1.2		1.3
	b 0.3	5.0	0.002	5.3	0.06	0.6		0.7
6 Rats	a 0.4	3.3		3.7				
	b 0.4	3.6	1.2	5.2	0.2	0.8	0.2	1.2
2 Rabbits	a	0.7		0.7	4.0	2.0		6.0
	b		0.7	0.7	16.5	0.4	0.7	17.6

* 400 mgm. DBA injected in each group of animals. Collection period of 6 days.

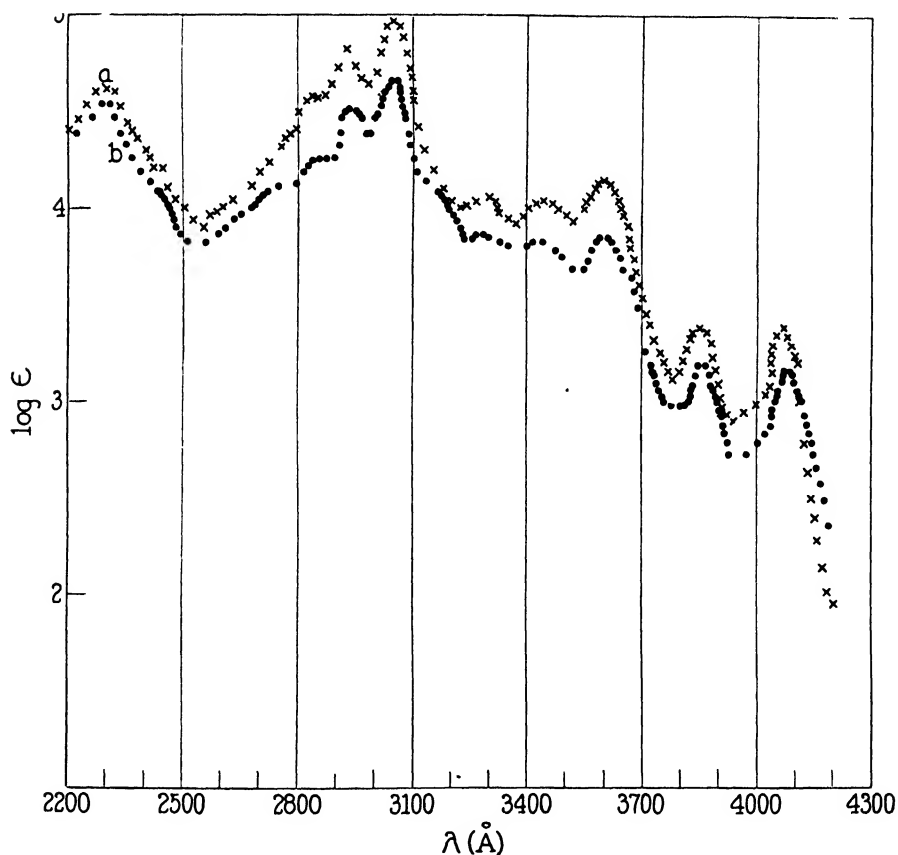


FIG. 4. Absorption curves of dihydroxydibenzanthracene: (a)—Isolated by Boyland and Levi (5). (b)—Isolated by Dobriner, Rhoads, and Lavin.

VI. The Comparative Ability of Different Species to Convert DBA

It has been demonstrated that different species show varying susceptibility in tumor development due to the administration of DBA (6). Furthermore, this group (12) and Boyland and Levi (5) have presented evidence that the phenolic metabolite of DBA excreted by rabbits does not produce tumors in mice. It was of interest, therefore, to investigate in various species the relative speed of conversion of DBA, as well as the rate of excretion of DBA and its conversion products.

Twenty mice, 6 rats, and 2 rabbits were injected with DBA dissolved in olive oil. Four hundred mgm. were administered to each group of animals. The urine and feces were collected for a 6-day period. The animals were killed; the total urines, feces, and intestinal tract contents of the animals in each species were worked up as described under METHODS. The experiment was repeated (Table IV).

A rough quantitative estimation of DBA present in the neutral fractions and of the phenolic conversion products present in the phenolic fractions was made. The sharp band of DBA present at 2,990 A. was diluted out. The series of dilutions were compared with similar dilution of standard solutions of pure DBA and of the phenolic conversion products. Since the neutral as well as the phenolic fractions from the three species contained a variable amount of background material which decreases the intensity of the bands, the quantitative estimation is very crude. Nevertheless, if large differences in the conversion and excretion rates are present, an indication may be obtained with regard to species differences.

As seen in Table IV, mice and rats excrete a relatively large amount of DBA as compared to rabbits. Mice and rats convert only a small amount of the injected DBA to a phenolic derivative as compared to rabbits. Moreover, the excretion and conversion of DBA is extraordinarily low in all species. These results are preliminary.

DISCUSSION

The value of the application of the spectroscopic method to the study of the metabolism of DBA is demonstrated by the following observations on the excreta of the DBA-injected animals: 1. In the crude ether extracts containing neutral substances, a series of bands identical in position with those of DBA was observed. 2. In the crude ether extracts containing the phenolic substances, a series of bands similar to but not in identical positions with the bands of DBA was seen. These bands suggest a phenolic conversion product of DBA. 3. The series of bands in the phenolic fraction obtained from the excreta of rats and of mice differed in their positions from those of the bands obtained from the same fraction of rabbit urine and feces.³ 4. The monkeys provided no evidence of excretion of DBA or an ether-soluble phenolic metabo-

lite of DBA. These observations on the crude fractions suggest that rats and mice excrete the same phenolic DBA derivative, but that this derivative is not identical with the phenolic conversion product excreted by rabbits. The sensitivity of this qualitative method is demonstrated by these observations inasmuch as the amount of DBA and its metabolites present in 72-hour urine and feces collections is extremely small (Fig. 4).

As pointed out in the first publication, there are factors which decrease the sensitivity of the spectroscopic methods. One of the most disturbing is the varying amount of background absorption which is given by all the fractions obtained from natural material. This background absorption renders invisible bands given by substances present in lower concentration. Thus, all the statements which concern negative findings must be interpreted as indicating that under the spectroscopic conditions applied no bands could be detected. This applies particularly to the study of tissue extracts. Further purification of the extracts by improved methods may provide evidence of the presence of traces of materials which give absorption in the ultraviolet region of the spectrum.

In the course of the isolation procedures, the spectroscopic method was extremely useful in its application to the fractions obtained by high vacuum distillation and chromatographic adsorption analysis. From the information thus obtained, it was possible to discard large amounts of residual material and to concentrate the small amounts of DBA metabolite present. The qualitative and quantitative spectroscopic analyses of the isolated compounds are shown in Figs. 3 and 2. Both spectroscopic methods show the identity of the absorption of the phenolic derivative obtained from rats and from mice, and the difference as compared with the phenolic derivative obtained from rabbits. The positions of the bands of the rabbit compound are identical with those of the dihydroxydibenzanthracene isolated by Boyland and Levi (Fig. 4).

The phenolic derivatives from the urine and feces of rabbits, rats, and mice were isolated from the phenolic ether-soluble fraction of the excreta by means of high vacuum distillation, chromatographic adsorption analysis, and recrystallization. Evidence from the melting points and mixed melting points of the phenolic derivatives of DBA demonstrates that rats and mice excrete a different compound than do rabbits. These facts agree with the evidence derived by the spectroscopic methods. Boyland and Levi (4, 5, 21) isolated dihydroxydibenzanthracene from the urines of rabbits fed with DBA. The chemical and physical properties of the compound isolated by us are the same as those of the compound isolated by Boyland and Levi. The positions of the hydroxyl groups in the compound excreted by rabbits have not yet been established. The problem is under investigation by Boyland and Levi (5) and by Cason and Fieser (8).

Fieser and Cason (7) synthesized 4',8'-dihydroxy-1,2,5,6-dibenzanthracene. From their evidence on melting points and mixed melting points, the

synthetic compound is identical with the phenolic compound isolated from the excreta of rats. The absorption spectrum of the synthetic compound as determined by Jones and Fieser, and by our group, is identical with the absorption of the DBA metabolite excreted by rats.

The possibility of the presence in the urine of conjugated derivatives of DBA and hydroxy DBA was investigated in the urine of rabbits, rats, and monkeys. The ether fractions obtained after hydrolysis and autoclaving of the urine did not yield additional DBA or phenolic derivatives as judged by the spectroscopic evidence. If phenolic derivatives of DBA were conjugated as glucuronides or as ethereal sulfates, the ether-soluble free compounds should have been present in these fractions. Occasionally, traces of the phenolic derivatives were observed after hydrolysis of the urine so that conjugation of this type cannot be entirely excluded. The possibility also exists that unstable ethereal sulfates or glucuronides are formed which hydrolyze immediately after acidification of the urine or during the extraction procedures. Boyland and Levi (5) have presented evidence of an increased excretion of ethereal sulfates following the feeding of DBA to rabbits. However, there is no proof that the increase is due to a conjugated DBA derivative. It is possible that, because of the toxic action of DBA, an increase of ethereal sulfates may result from substances other than metabolites of this hydrocarbon.

The possibility of the formation of a DBA mercapturic acid was investigated. Since mercapturic acids are rarely ether-soluble, chloroform and butyl alcohol extractions of rabbit and rat urines were made. No indications, either chemical or spectroscopic, of a DBA mercapturic acid were observed. Furthermore, following the hydrolysis or autoclaving procedures, no free DBA was observed spectroscopically. From the data, it appears that the formation of conjugated derivatives of DBA or its hydroxy derivatives is not one of the principal metabolic pathways for the handling of DBA.

Aromatic unsubstituted hydrocarbons are hydroxylated in many instances in the course of their metabolism. The symmetrical configuration of the phenolic dihydroxy conversion product excreted by rats is an interesting metabolic phenomenon. Hydrocarbons of more than one ring system are metabolized to mono-hydroxylated derivatives (naphthalene) or to dihydroxy dihydro derivatives in neighboring positions (anthracene). Boyland and Levi (2, 3) showed that rabbits and rats fed with anthracene excrete optical isomers of 1,2-dihydroxy-1,2-dihydroanthracene. Wiley (25) gave evidence for the hydroxylation of β -naphthylamine in neighboring positions in dogs, whereas Dobriner, Hofmann, and Rhoads (13) have shown that rats, rabbits, and monkeys excrete a β -naphthylamine metabolite which is hydroxylated in the 6- or symmetrical position. How far species differences are responsible for hydroxylation in neighboring or symmetrical positions remains to be demonstrated.

Although only a few studies on the metabolism of carcinogenic hydrocarbons

are available, only in the case of DBA has a phenolic derivative been isolated. Evidence indicates that methylcholanthrene (15), 3,4,5,6-dibenzcarbazole (5) and benzpyrene (9, 10) probably are metabolized to phenolic compounds. The primary formation of alcoholic dihydroxy dihydro derivatives in neighboring positions and the secondary splitting off of water with the formation of a phenol as possibly in the case of naphthalene metabolism, must be considered. Furthermore, the biochemical and biological significance of the formation *in vivo* of phenolic derivatives of carcinogenic hydrocarbons must be established. Some information is available in regard to the carcinogenic activity of synthetic phenolic and alcoholic derivatives of very active carcinogens. These compounds show very little or no carcinogenic activity (1, 17, 16, 23, 24, 18-20).

The difference in metabolism of DBA among various species which results in the production of dihydroxy derivatives of different configuration is of great interest not only from the metabolic point of view but also from the point of view of carcinogenic activity.

Since DBA produces tumors in mice (11), and to a lesser extent in rats, and not at all in rabbits (6), it is of interest to know whether the carcinogenic properties of DBA are dependent upon the intermediary metabolism or upon the final conversion of this compound. This investigation has demonstrated that various species metabolize DBA differently and has raised the question of the carcinogenic power of the DBA metabolites produced by the different species.

The phenolic derivative produced by rabbits was injected into mice and was found to be noncarcinogenic. A control experiment in which DBA was injected in the same amount into litter mates of the mice showed that this strain of mice was highly susceptible to tumor formation caused by DBA. The phenolic derivative produced by the rabbit was injected only into a small number of mice. A larger series of animals and the administration of larger amounts is desirable. Boyland tested 10 mice by painting the skin twice weekly with the dihydroxydibenzanthracene derivative which he isolated from rabbits. He also injected 10 mice twice weekly with 1 mgm. each of the phenolic derivative. None of the mice showed tumor formation, thus confirming the findings of this group. It will be of great interest to know whether the phenolic derivative produced by rats and mice will have the same lack of carcinogenic activity.⁵ The decrease or lack of carcinogenic power of synthetic hydroxylated hydrocarbons has been discussed previously. There

⁵ Since this manuscript was submitted, Dunlap and Warren (Cancer Research, 1: 953-954. 1941) have reported that 4',8'-dihydroxy-1,2,5,6-dibenzanthracene, the phenolic derivative excreted by rats and mice, did not produce tumors in mice when tested over a period of 8 months.

is no doubt that both *in vivo* and *in vitro*, hydroxylation renders strong carcinogens less or noncarcinogenic. The question still remains as to why the carcinogenic potency of the same hydrocarbon is different in different species. There is some evidence that only a very small amount of DBA injected in olive oil is metabolized to a noncarcinogenic phenolic derivative. A large amount of the DBA remains in the animal. Lorenz and Stewart (22) have reported that DBA can be recovered only in very small amounts from the feces of mice which have been fed the hydrocarbon. Studies are in progress which, it is hoped, will provide more information concerning the qualitative and quantitative aspects of DBA metabolism in different species.

SUMMARY

1. By a combination of chemical fractionation and qualitative spectroscopic methods the fate of dibenzanthracene was investigated in the excreta and certain tissues of mice, rats, rabbits, dogs, and monkeys.

2. The spectroscopic investigations gave evidence for the presence of phenolic derivatives of dibenzanthracene in the excreta of mice, rats, rabbits, and dogs. No metabolite of dibenzanthracene was suggested in the excreta of monkeys.

3. The spectroscopic investigation suggested that the phenolic derivatives of dibenzanthracene are identical in mice and rats and differed from the phenolic metabolite excreted by rabbits.

4. No evidence was obtained for the excretion of a conjugated derivative of dibenzanthracene in any of the species studied.

5. Small amounts of dibenzanthracene are excreted in the urine and feces of mice, rats, and rabbits.

6. A preliminary quantitative estimation of the comparative ability of different species to convert dibenzanthracene shows that mice and rats convert only a small amount of the injected dibenzanthracene to the phenolic derivative as compared to rabbits.

7. The phenolic derivatives of dibenzanthracene excreted by mice, rats, and rabbits were isolated. The phenolic metabolites of mice and rats are identical with 4',8'-dihydroxydibenzanthracene synthesized by Fieser and Cason.

8. The dihydroxydibenzanthracene produced by rabbits did not show carcinogenic activity when injected in mice.

9. The chemical and biological significance of the conversion of dibenzanthracene to phenolic derivatives is discussed.

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THE EFFECT OF INTRAPERITONEAL INJECTIONS OF CARBON INK ON THE COURSE OF PLASMODIUM LOPHURAE INFECTIONS IN CHICKENS

By WILLIAM TRAGER

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for
Medical Research, Princeton, New Jersey)

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Coggeshall (1938), in his original work with *Plasmodium lophurae*, observed that, while severe infections could be readily produced in very young chicks, even very light infections could be produced only with difficulty in older chickens. The existence of this age immunity has been confirmed by Taliaferro and Taliaferro (1940) and in the present work. The exposure of older chickens to very low temperatures, their maintenance on a diet low in vitamins of the B₂ group, or their treatment with phenylhydrazine hydrochloride all failed to diminish their resistance to infection with *P. lophurae*. The inoculation of large doses of carbon ink did, however, markedly increase the susceptibility of 4 to 9-week-old chickens.

Materials and Methods

A strain of *Plasmodium lophurae*, obtained through the kindness of Dr. L. T. Coggeshall, was maintained by passage every 6 days into 2 to 3-day-old chicks. At each passage heparinized blood from the heart of a heavily infected chick was inoculated intracerebrally into the fresh chicks in a dose of 0.05 cc.

The larger chickens used for the experimental work were 4 to 9 weeks old and weighed about 200 to 350 grams. They were inoculated intracardially with about 0.8 cc. of pooled heparinized blood from several heavily infected young chicks. In some of the experiments the erythrocytes per cu. mm. and the number of parasites per 10,000 erythrocytes of the blood used for inoculation were determined. Chickens treated with ink and suitable control chickens of about the same age and weight were inoculated at the same time with the same infected blood. The controls were left otherwise untreated, except in one experiment in which they received 0.85 per cent sodium chloride solution into the posterior peritoneal cavity. Counts were made on dried Giemsa-stained blood films, prepared from the inoculated chickens at intervals during the course of the infection until the parasite number had passed its peak. The number of parasites per 10,000 red blood cells was determined by counting a sufficient number of erythrocytes in the manner described by Gingrich (1932).

The carbon ink preparation used was a 15 per cent by volume dilution of *Higgins Eternal* black writing ink in distilled water, with sodium chloride added to give a concentration of 0.85 per cent. The mixture was filtered through coarse fluted paper and autoclaved. The diluted ink was injected into the ventral right portion of the posterior peritoneal (pelvic) cavity of the treated chickens, in a dose at each inoculation of 5 cc. per 100 grams of body weight. The chickens received ink on the day before inoculation with the malaria parasites, and for several days thereafter. There was considerable individual variation in the degree to which these large doses of ink were tolerated, but robust-appearing young roosters usually survived the treatment in reasonably good condition. Chickens receiving ink into the anterior peritoneal cavity rapidly became very weak and died after one or two injections. Autopsy revealed extensive damage to the liver. In several experiments, smaller doses of ink were administered intracardially.

Pieces of liver, spleen, and bone marrow from some of the experimental chickens were fixed in Zenker's, sectioned and stained by Wolbach's (1919) Giemsa method.

RESULTS

The results of seven representative experiments are presented in Tables 1 and 2. Chickens 1 to 9, inclusive, were White Leghorns, while all the others were Rhode Island Reds having a slight admixture of Plymouth Rock blood. It is apparent from the data that the largest parasite numbers occurred among the chickens receiving intraperitoneal injections of ink; that such chickens usually showed a considerably larger peak parasite number than corresponding control chickens; that the peak occurred from 1 to several days later in the course of the infection in treated than in untreated chickens; and that appreciable numbers of parasites were present in the blood of treated chickens for a longer period than in the blood of untreated chickens. Thus in experiment 1 (figure 1), the untreated chickens showed peaks of 960 and 1,740 parasites on the third and the fourth day; those receiving intracardial injections of ink showed peaks of 1,380, 2,420, and 1,400 parasites on the third, fourth, and third days, respectively; while those receiving intraperitoneal injections of ink showed peaks of 3,740, 7,940, 1,520, and 2,620 all on the fifth day. In the untreated chickens and those injected intracardially with ink, very few parasites remained on the fifth and sixth days, while in those injected intraperitoneally there were still 500 or more per 10,000 red cells on the sixth day.

Some of the other experiments given in tables 1 and 2 show equally striking results. But in several experiments a chicken receiving ink intraperitoneally would develop only a very mild infection. Such a result is illustrated in experiment 4. Chicken 17, which was injected intraperitoneally with ink, showed a much heavier infection than either of the two controls. But chicken

TABLE 1

Parasite Counts on Chickens Treated with Diluted Carbon Ink and on Untreated Controls

Exper. No.	Chicken No.	Weight (gm.)	Treatment*	Dose of parasites	Parasites per 10,000 erythrocytes on days after inoculation					
					0	1	3	4	5	6
1	1	229	Ink I.C. on -1, 1, 3	0.7 cc. heavily infected blood	—	250	1380	1280	65	—
	2	166	" " " -1, 1	0.7 " " " "	—	560	2060	2420	920	0
	3	238	" " " -1, 1, 3	0.7 " " " "	—	235	1440	900	0	—
	4	202	None	0.7 " " " "	—	310	1700	1740	0	—
	5	205	"	0.7 " " " "	—	105	960	630	0	—
	6	202	Ink I.P. on -1, 0, 1, 2, 3, 4	0.7 " " " "	—	305	1900	2880	3740	610
	7	174	" " " -1, 0, 1, 2, 3	0.7 " " " "	—	390	2620	4840	7940	Died
	8	227	" " " -1, 0, 1, 2, 3, 4, 5	0.7 " " " "	—	160	1520	1420	1520	560
	9	218	" " " -1, 0, 1, 2, 3, 4, 5	0.7 " " " "	—	180	1380	2160	2620	1280
2	10	238	None	0.8 " " " "	—	295	1780	2080	2820	1460
	11	262	Ink I.P. on -1, 0, 1, 2, 3, 4	0.8 " " " "	—	145	880	2100	4120	5820
	12	324	" " " -1, 0, 1, 2, 3, 4	0.8 " " " "	—	160	1620	2240	4340	4800
3	13	384	None	0.8 " " " "	—	260	710	1020	105	—
	14	409	"	0.8 " " " "	—	250	800	450	65	—
	15	354	Ink I.P. on -1, 0, 1, 2, 3, 4	0.8 " " " "	—	240	1760	2200	6360	—
	16	485	" " " -1, 0, 1, 2, 3, 4	0.8 " " " "	—	185	510	900	670	—
4	17	224	" " " -1, 0, 1, 2, 3, 4	116.5×10^6	—	350	1440	1960	2980	—
	18	215	None	116.5×10^6	—	225	1460	800	0	—
	19	256	Ink I.P. on -1, 0, 1, 2, 3, 4	116.5×10^6	—	165	300	600	340	—
	20	260	None	116.5×10^6	—	165	550	45	0	—
5	21	340	Ink I.P. on -1, 0, 1, 2, 3, 4	753×10^6	—	200	880	1280	940	—
	22	232	" " " -1, 0, 1, 2, 3, 4	753×10^6	—	245	470	530	230	—
	23	285	" " " -1, 0, 1, 2, 3, 4	753×10^6	—	215	1440	2440	2980	—
	24	217	None	753×10^6	—	370	1960	860	0	—
	25	373	"	753×10^6	—	170	800	160	0	—
6	26	250	Ink I.P. on -1, 0, 1, 3	834×10^6	130	240	390	650	190	—
	27	192	" " " -1, 0, 1, 3	834×10^6	107	450	1600	1900	1700	—
	28	173	None	834×10^6	215	450	620	220	0	—
	29	180	"	834×10^6	275	450	2720	2780	2380	—

* Ink I.C. means that the chicken was inoculated intracardially with 5 cc. per 100 gm. body weight of a .15% dilution of carbon ink in 0.85% salt solution.

Ink I.P. means that the chicken was inoculated intraperitoneally with 5 cc. per 100 gm. body weight of the same ink dilution.

The numbers refer to the days on which the dose of ink was administered, the day when the parasites were inoculated being considered as day 0. Thus "Ink I.P. on -1, 0, 1, 2, 3" means that the chicken received intraperitoneally a dose of 5 cc. of the diluted ink per 100 gm. body weight on the day before inoculation of parasites, on the day of inoculation, and on each of the following 3 days.

19, also injected intraperitoneally with ink, showed a peak parasite number about the same as that of control chicken 20, which had the same weight, and lower than that of control chicken 18, which weighed somewhat less. It is noteworthy, however, that in both control chickens the peak parasite number

was reached on the third day of the infection and very few parasites were present by the fifth day, whereas in chicken 19 the peak was reached on the fourth day and on the fifth day there were still present 340 parasites per 10,000 red cells.

In only two experiments, one of which is shown in table 1 (experiment 6), did one of the untreated control chickens exhibit a heavier and more prolonged infection than any of the chickens treated with ink. In experiment 6, both of the control chickens weighed less than the treated ones and showed about twice as many parasites within a few minutes after inoculation. Both of the treated chickens had mild infections, with the peak parasite number on the fourth day and with many parasites still present on the fifth day. One of the control chickens had the usual type of infection with the peak on the third day and

TABLE 2

Exper. 7.—Parasite and erythrocyte counts on chickens treated with diluted carbon ink (nos. 30, 31), on chickens treated with salt solution (nos. 32, 33), and on untreated chickens (nos. 34, 35). Each chicken was inoculated intracardially with $1,039 \times 10^6$ parasites. The ink and salt solutions were injected I.P. on days -1, 0, 1, 2, 3 (see footnote, Table 1).

Chicken No.	Weight (gm.)	Parasites per 10,000 erythrocytes on days after inoculation									Erythrocytes per cu. mm. ($\times 10^6$) on days after inoculation								Parasites per cu. mm. ($\times 10^4$) on days after inoculation							
		0	1	2	3	4	5	6	7	8	1	3	4	5	6	7	8	1	3	4	5	6	7	8		
30	270	65	230	310	780	1080	2440	3680	1400	540	2.0	1.9	2.2	2.0	1.7	1.4	0.9	0.46	1.48	2.38	4.88	6.26	1.96	0.49		
31	267	80	80	130	530	820	1560	1300	1800	230	2.1	2.2	2.3	2.3	1.7	1.5	1.0	0.17	1.17	1.89	3.59	2.21	2.70	0.23		
32	272	185	165	285	600	400	70	0	—	—	1.8	1.7	1.6	1.7	1.8	—	—	0.30	1.02	0.64	0.12	0	—	—		
33	269	155	115	105	290	50	0	—	—	—	2.0	0.9	1.3	1.4	1.7	—	—	0.23	0.26	0.07	0	—	—	—		
34	253	180	220	350	1000	140	0	—	—	—	1.3	1.3	1.6	1.8	—	—	—	0.44*	1.30	0.18	0	—	—	—		
35	260	100	190	460	730	265	0	—	—	—	1.3	1.4	1.6	1.7	—	—	—	0.38*	0.95	0.37	0	—	—	—		

* Count based on assumption of 2 million erythrocytes per cu. mm.

very few parasites left on the fifth day. But the other control chicken (29) showed a heavy infection, with the peak on the fourth day and very many parasites still present on the fifth day.

In the course of the experiments it was observed that the blood of the infected chickens appeared thin and watery on the day following the peak parasite number, and that many young red blood cells were present. Large numbers of young erythrocytes almost always appeared from one to several days sooner in the control than in the treated chickens. In order to get more precise information on this matter, an experiment (7, table 2) was performed in which erythrocyte as well as parasite counts were made. It is apparent that the 4 control chickens became markedly anemic by the third day and that the blood was being regenerated on the fifth and sixth days. In the two chickens which received ink intraperitoneally there was no marked reduction in red blood cells until the sixth day, and the minimum was not reached until the

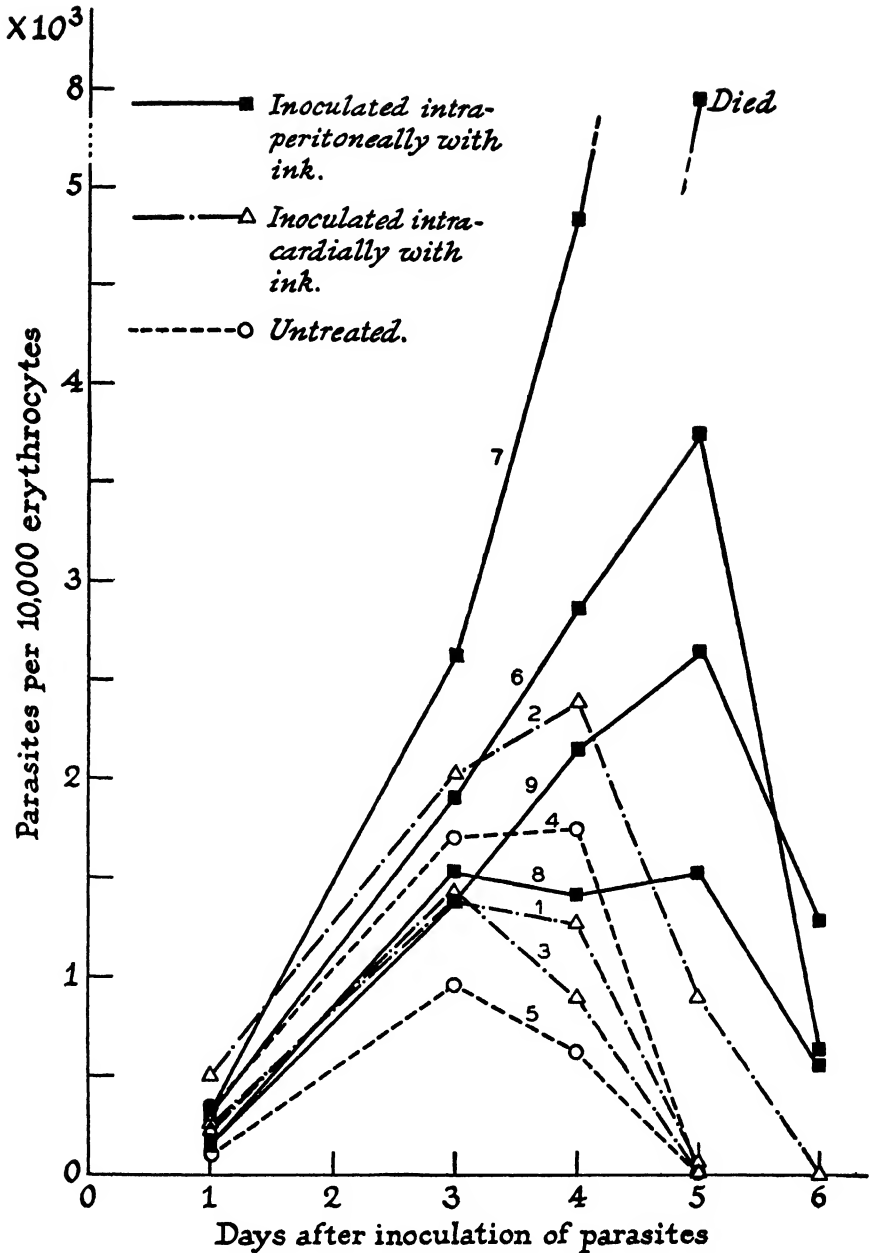


FIG. 1. The course of *P. lophurae* infections in control chickens and in chickens receiving carbon ink intracardially and intraperitoneally. (Experiment 1, number on each curve corresponds to chicken number.)

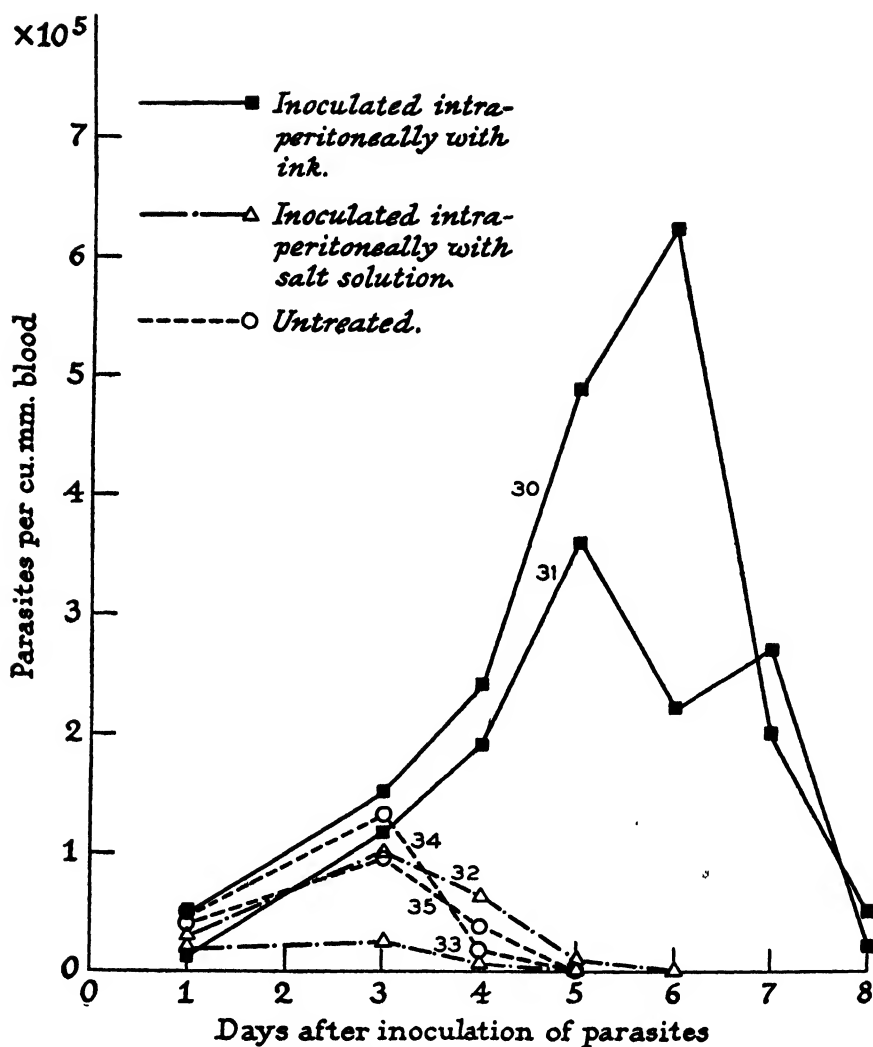


FIG. 2. Absolute parasite numbers during *P. lophuræ* infections in control chickens, in chickens receiving salt solution, and in chickens receiving carbon ink intra-peritoneally. (Experiment 7, number on each curve corresponds to chicken number.)

eight day, when the experiment was discontinued. In this experiment the number of parasites per cu. mm. of blood was determined, with the same type of result as was obtained in this and the other experiments on the basis of the relative number of parasites (table 2, figure 2).

At autopsy, most of the control chickens showed no obvious changes from

the normal, while in those few which had developed a relatively severe infection the spleen was slightly enlarged and darkened and the liver was brown. In sections of liver and spleen from chickens of the latter group there could be seen numerous clusters of small pigment granules, but very few such granules could be found in the bone marrow. This is in accord with the finding in all types of malaria (Taliaferro and Mulligan, 1937) that, of the 3 organs most important in defense against the infection, the spleen and liver show much more phagocytic activity than the bone marrow. In chickens treated with carbon ink intraperitoneally, there might or might not be a considerable accumulation of fluid in the pelvic cavity. There was always a friable black deposit lining the right ventral portion of the pelvic cavity and the peritoneal membranes were thickened. The spleen was dark red to black in color, while the liver varied from gray to black. The bone marrow rarely appeared darker than normal. Sections of spleen and liver of such chickens showed very many clusters of dark granules, and the individual macrophages were packed full of this material (whereas in the untreated but relatively heavily infected chickens the macrophages contained scattered discrete granules). The bone marrow again contained much less dark material than the spleen and liver. Chickens which received carbon ink intracardially and which developed only comparatively light infections, showed at autopsy a black liver and spleen and very dark bone marrow. In sections of liver and spleen there were large confluent masses of dark material, and in sections of bone marrow there were many scattered macrophages full of dark granules.

DISCUSSION

If chicks about 2 to 3 days old are inoculated intraperitoneally, intracardially, or intracerebrally with a large dose of *Plasmodium lophurae*, they develop a more or less intense infection with a peak parasite number on the fifth to seventh day, followed by a rapid reduction in the number of circulating parasites (see also Coggeshall, 1938). In chickens more than 3 weeks old, as shown by the results of Taliaferro and Taliaferro (1940) and of the work here reported, the peak parasite number usually occurs on the third day or sooner, and is followed, as in the younger chicks, by a swift reduction in the number of parasites in the blood. But in the older chickens which received maximal doses of carbon ink into the posterior peritoneal cavity, the parasites continued to multiply beyond the third day and reached their peak on the fifth day, or at almost the same time as in the younger chicks. In most of the experiments the administration of the ink had to be discontinued by the fourth day after infection in order to prevent the chicken from becoming too weak. However, in one experiment in which it was possible to continue the ink inoculations through the eighth day the parasite number nevertheless reached its peak on the sixth day and then rapidly declined. In any case, older chickens are ap-

parently able to develop an *effective* resistance to the parasite at least 2 to 3 days sooner than the very young chicks, and this ability is interfered with by the ink inoculations. Hence it seems justifiable to conclude that the ink injections upset the mechanism which is responsible, in this infection, for the age immunity.

That this mechanism might be a more efficiently functioning lymphoid-macrophage (reticulo-endothelial) system appears at first sight a likely guess. This system is responsible for both natural and acquired immunity to malaria (Taliaferro and Mulligan, 1937). It is well known that with excessive doses of carbon ink, or certain other substances, the cells of this system can be blockaded and animals made more susceptible to certain infections (Cannon and McClelland, 1929) and less capable of producing antibodies (Gay and Clark, 1924; Isaacs, 1925). There are few published reports concerning the effects of blockade of the lymphoid-macrophage system on malarial infections, in contrast to the numerous papers dealing with decreased resistance after splenectomy. Kritschewski and Meerson (1932) found that the intramuscular injection of trypan blue shortened the prepatent period of *Plasmodium praecox* in siskins. The effect here must have been exerted on the natural resistance mechanism which is active before any appreciable degree of acquired immunity has appeared. Malamos (1934) observed that intravenous injection of *Macacus cynomolgus* monkeys with carbon ink, or intraperitoneal injection with trypan blue, just before their inoculation with *Plasmodium knowlesi*, rendered the ensuing malarial infection fatal. Such injections had no effect on the chronic stage of the disease, although splenectomy during the chronic stage produced severe relapse. Findlay (1933), however, states that, while in resistant birds which are superinfected with malarial parasites the parasites disappear within 48 hours, in similar birds blockaded with carbon ink or trypan blue the parasites remain in the peripheral blood for 4 or 5 days. Gingrich (1934) found that blockade with foreign red cells of the macrophage system of canaries infected with *Plasmodium cathemerium* had no effect during the acute stage of the disease, but did have a marked effect just after the crisis and during the latent period.

The data on *P. lophurae* infections reported in the present paper suggest that the effect of the ink is exerted primarily by delaying the appearance of acquired immunity, rather than by interfering with the natural resistance to the infection. During the first 3 days the parasites increase at the same rate in treated and untreated chickens. In the latter an effective acquired immunity appears by the third or fourth day, while in the former it does not appear until the fifth or sixth day, as in very young chicks receiving comparable large doses of parasites.

There is some evidence derived from various types of experiments that the reticulo-endothelial system of very young animals is functionally immature

(Baumgartner, 1934; Dingle, Meyer, and Gustus, 1936). Quite recently, Culbertson and Wotton (1939) and Kolodny (1940) have shown that very young rats are unable to produce effective amounts of antibodies against *Trypanosoma lewisi* and *T. cruzi*. One may consider that the ink treatment of older chickens sufficiently disturbs the function of the lymphoid-macrophage system as to render it no more effective in the production of acquired immunity against *P. lophurae* than the immature system of very young chicks.

One peculiar fact which does not fit in with the mechanism outlined above is the failure of intracardial injections of ink to produce any effect comparable to that produced by the injections into the posterior peritoneal cavity (experiment 1, and two other similar experiments not tabulated). The amount of ink was indeed smaller, but the extent of blockade, as judged histologically, was vastly greater in the chickens injected intracardially than in those injected intraperitoneally. It is also interesting and worthy of emphasis that the injections of ink into the anterior peritoneal cavity, a procedure which damaged the liver and severely weakened the chicken, did not have any effect on the course of the *Plasmodium lophurae* infection in those few chickens which survived long enough for the observations to be made.

SUMMARY

In 4 to 9-week-old chickens inoculated with large doses of *Plasmodium lophurae*, the parasite number usually reached a peak on the third day and then rapidly decreased. In similar chickens which received carbon ink into the posterior peritoneal cavity the parasite number continued to increase beyond the third day, reached a peak, usually higher than in control chickens, on the fifth day, and then fell off less rapidly than in untreated chickens. The course of the infection in the treated chickens resembled that seen in very young chicks, suggesting that the ink treatment interfered with the mechanism responsible for the age immunity observed in untreated older chickens.

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SIMULTANEOUS INOCULATION OF VARIOLA AND VACCINIA VIRUSES IN EMBRYONATED EGGS

By JOHN B. NELSON

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

In connection with some observations on the stability of variola virus it became of interest to determine the behavior of a small amount of vaccinia virus in the presence of a large amount of variola virus on inoculation in embryonated eggs. The relation of the ensuing findings to the problem of mutability as it pertains to variola virus will be considered elsewhere. The results are reported here simply as one example of the activity of 2 infective agents inoculated simultaneously in fertile eggs, a phase of that method on which there appears to be little data.

Two bacteriologically sterile strains of variola virus (Minnesota and Chinese) which had been carried through 202 and 38 transfers, respectively, in embryonated eggs were used, together with a single strain of vaccinia virus (New York City Board of Health) maintained by occasional egg transfer. The 2 variola strains react identically in 10-day fertile hen's eggs following implantation on the retracted chorioallantoic membrane. By the 3rd day a 10% membrane suspension of either strain produces a thickened confluent area at the site of inoculation but does not affect the embryo. The strain of vaccinia virus behaves very differently, and the 2 virus species may generally be identified by inspection. Eggs inoculated with this strain of vaccinia show a much thinner area of reaction in the membrane by the 3rd day, the result of rapid cell degeneration, and usually a dead embryo.

Saline suspensions were prepared containing variola virus diluted 10^1 and vaccinia virus diluted 10^6 , the dilution figure being based on the wet weight of small membrane sections removed aseptically on the 3rd day from 10-day hen's eggs inoculated with the respective viruses. Prior to dilution the membrane sections were finely ground in glass tissue grinders. Similar dilutions, serving as activity controls, were also set up with each virus alone. Two or 3 embryonated eggs were then inoculated with approximately 0.5 cc. of the virus mixtures and the individual suspensions. After incubation at 37°C for 3 days the inoculated eggs were opened for examination, and approximately 10% suspensions prepared from the membranes. Three to 5 successive egg transfers were subsequently made with each series.

The detailed results of one experiment are as follows: Two embryonated eggs opened on the 3rd day after inoculation with a mixture containing Chinese

variola virus diluted 10^1 and vaccinia virus diluted 10^6 showed active embryos and the thickened membrane characteristic of variola, an identical reaction being obtained with the 10^1 dilution of variola virus alone. Eggs inoculated with the 10^6 dilution of vaccinia virus alone also showed active embryos with scattered discrete foci in the membrane, indicative of a highly diluted suspension of that virus. A 2nd transfer was made with each series, using 10% membrane suspensions. In the eggs inoculated with the virus mixture the embryos were inactive and the membranes moderately thickened, suggestive of an intermediate reaction. The eggs inoculated with variola virus alone showed the customary thickened membrane and active embryos; whereas in those inoculated with vaccinia virus alone the membrane was thin and the embryos dead, the characteristic reaction of this virus in low dilution. In the 3rd passage the individual virus suspensions behaved in the usual manner, but the mixture resulted in death of the embryos and thinning of the membrane at the site of inoculation, the typical manifestations of vaccinia virus alone.

Essentially the same results were obtained in 2 additional experiments using similar mixtures of vaccinia virus and the Chinese strain of variola, the former being masked in the first transfer but predominating by the 3rd. The procedure was repeated with mixtures of the same vaccinia virus and the Minnesota strain of variola. In the first test there was no apparent development of the vaccinia virus through the 5th transfer although it was demonstrable in the 10^6 dilution prior to mixing. In 2 subsequent tests, however, the preceding results were duplicated, vaccinia virus multiplying in the presence of a high concentration of variola virus and predominating by the 3rd transfer. It may be added that in the long series of egg inoculations made with the Minnesota strain of variola virus, since it was isolated in 1938, its behavior in embryonated eggs has remained constant with no departure in the direction of vaccinia virus.

Egg membranes from the 3rd transfer of the Chinese variola-vaccinia virus mixture and the 4th transfer of the mixture with the Minnesota strain were tested in dilutions through 10^7 by the inoculation of scarified areas in the skin of susceptible rabbits. A graded response typical of vaccinia virus was obtained through a dilution of 10^6 . The corresponding suspensions of the two variola strains alone behaved in the customary way, producing no macroscopic reaction in the skin of inoculated rabbits. Cutaneous inoculation of the monkey to determine the presence of variola virus in membranes inoculated with the virus mixtures was not carried out.

Summary. Mixtures of variola virus diluted 10^1 and vaccinia virus diluted 10^6 were inoculated in embryonated eggs and followed by 3 or more successive transfers. In 5 of the 6 tests the highly diluted vaccinia virus was masked in the 1st transfer but subsequently multiplied and predominated by the 3rd. Two of the transferred mixtures on inoculation in rabbits produced a typical vaccinal reaction in dilutions through 10^6 .

CHANGES IN HORMONE CONTENT OF THE FEMALE RABBIT HYPOPHYSIS AFTER MATING

By JOHN A. SAXTON, JR., AND HARRY S. N. GREENE

*(From the Department of Animal and Plant Pathology of The Rockefeller Institute
for Medical Research, Princeton, New Jersey)*

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Experiments cited by Hill (1) have shown that in the rabbit ovulation is brought about by a neuro-secretory mechanism. Impulses induced by copulation stimulate the anterior hypophysis to discharge gonadotropic hormones, which may then act upon the ovary to produce ovulation in from ten to twelve hours. Ovulation will not take place if the hypophysis is removed within one hour after mating (2). Hill (1) has shown that there is a progressive decrease in gonadotropic potency of the rabbit hypophysis following mating, and that the lowest level of potency is reached in about 24 hours. The discharge from the hypophysis would thus appear to continue beyond the period required for ovulation. The hormones released in the postovulatory period may therefore be available for the production of corpora lutea and perhaps for other processes. Hill's method of analysis, using the female rabbit in estrus as the test animal, gives a measure of the gonadotropic potency only, and does not permit an estimation of the relative amounts of other hormones discharged simultaneously.

The method of Loeb (3), using the immature female guinea pig as the test animal, enables an assay of the content of gonadotropic (follicle-stimulating and luteinizing), thyrotropic and adrenal cortex-stimulating hormones of the rabbit hypophysis (4). This method has been used in a study of the hypophyses of mated female rabbits in order to determine indirectly the composition of the discharge of hormones after mating.

Materials and Methods

The hypophyses were obtained from 7 female rabbits of a Belgian-English hybrid strain, aged 4 to 6 months, and killed 4 to 48 hours after mating. Ovulation was confirmed at autopsy in all but one animal, which was killed 4 hours after mating. In this case, congestion of the ovaries and enlargement of the follicles was taken as evidence that ovulation would have occurred at the expected time.

The method of analysis has been described in detail elsewhere (4). Five hypophyses, from rabbits killed 4, 24, 24, 28 and 48 hours after mating, were analyzed separately by subcutaneous implantation of one-quarter of the gland daily for 4 days into immature female guinea pigs. One experiment was per-

formed using the hypophyses of two rabbits killed 24 hours after mating, one half gland being implanted daily into a guinea pig. As a control to this experiment, the hypophyses of two unmated females, litter mates of these rabbits, were implanted in the amount of one half gland daily into a single guinea pig.

In each case the test animals were killed 24 hours after the fourth implant. At autopsy both ovaries and the left adrenal of each guinea pig were weighed, and the gross appearance of the thyroid, uterus and vagina were noted. Microscopic sections of both ovaries, the left adrenal and one thyroid lobe were prepared. The standards previously described (4) were used in evaluating the changes in the test animals. Thus the effects of mating on the hormone content of the hypophysis could be determined by a comparison of these results with test animals receiving implants of single hypophyses from normal female rabbits. The average values for six untreated guinea pigs and the changes resulting from implantation of glands from normal Belgian-English females of the same age are included from the previous report for comparison.

RESULTS

The effects of implanting single rabbit hypophyses into immature female guinea pigs are shown in table 1. Hypophyses obtained within 48 hours after mating produced less follicle maturation and less luteinization in the ovaries of the test animals than did hypophyses from normal unmated female rabbits of the same age and strain. This indicates that both follicle-stimulating and luteinizing hormones are discharged during the postcopulatory period. The follicle-stimulating and luteinizing effects were lowest at 24 and 28 hours after mating, corresponding to the period of lowest gonadotropic potency observed by Hill (1). At 4 hours after mating there was less depletion than at 24 or 28 hours, and at 48 hours the gonadotropic effects appeared to be partially restored. Hypophyses from mated rabbits as compared with unmated ones produced a relatively slight increase in weight of the adrenals over the average weight of adrenals in untreated guinea pigs. However, the number of mitoses in representative sections was increased in all cases, and in this respect the adrenal stimulation was similar to that found after implanting glands from normal rabbits. Single hypophyses from rabbits 24 and 28 hours after mating produced no stimulation of the thyroid, in contrast to slight or moderate stimulation usually produced by single hypophyses from normal unmated female rabbits.

To confirm the decrease in thyrotropic potency following mating, larger amounts of hypophyseal tissue were used. In table 2 are shown the effects of implanting twice the amount from mated and unmated rabbits. Hypophyses from two unmated rabbits produced marked thyroid stimulation in the test animal in contrast to slight stimulation produced by hypophyses from two litter mates of these rabbits 24 hours after mating. Luteinization was greater

TABLE 1

Effects in Guinea Pigs of Implantation of Individual Hypophyses from 5 Mated Female Rabbits

Experiment	Av. Wt. of Hypophysis	Av. Initial and Final Wt.	Condition of Uterus and Vagina	Ovaries			Thyroid Stimulation	Left Adrenal	
				Av. wt.	Follicle maturation	Luteinization		Av. wt.	Mitoses/section, av.
*Control, 6 normal untreated guinea pigs	—	189	Ut. thin; vag. closed	16	0	0	0	41	10
*Hypophyses from 8 normal female rabbits	39	181-181	Ut. enlarged; vag. open	29	++	+++	+	55	20
Hypophysis from 1 female 4 hr. after mating	38	175-155	Ut. enlarged; vag. open	26	+±	+	+	46	123
Hypophyses from 2 females 24 hr. after mating	38	183-190	Ut. enlarged; vag. open	22	+±	±	0	48	20
Hypophysis from 1 female 28 hr. after mating	31	180-188	Ut. enlarged; vag. open	20	+	±	0	37	18
Hypophysis from 1 female 48 hr. after mating	32	175-175	Ut. thin; vag. closed	41	+±	+	+	46	18

* These experiments are from a previous paper (4).

TABLE 2

Effects in Guinea Pigs of Implantation of Hypophyses from 2 Mated Female Rabbits, and of Hypophyses from 2 Unmated Female Rabbits

Experiment	Av. Wt. of Hypophysis	Initial and Final Wt.	Condition of Uterus and Vagina	Ovaries			Thyroid Stimulation	Left Adrenal	
				Av. wt.	Follicle maturation	Luteinization		Wt.	Mitoses/section, av.
Hypophyses from 2 unmated rabbits implanted into 1 guinea pig	49	175-200	Ut. enlarged; vag. closed	21	++	+++±	+++±	46	24
Hypophyses from 2 rabbits 24 hr. after mating	47	170-185	Ut. enlarged; vag. open	31	+++	++	+	70	36

in the ovaries of the test animal receiving hypophyses from the unmated rabbits, but follicle maturation was less. Microscopic examination of the ovaries showed that in this case the excessive premature luteinization had largely prevented follicular growth and maturation. In each case the adrenal showed evidence of stimulation, but was more marked in the guinea pig receiving the hypophyses from the mated rabbits.

COMMENT

The results so far as changes in gonadotropic and thyrotropic effects are concerned, were consistent and appear to be significant in spite of the small number of cases. These experiments confirm the observations of Hill (1), that there is a decrease in gonadotropic potency of the female rabbit hypophysis after mating. Furthermore, they show that both the follicle-stimulating and the luteinizing hormones are decreased in the hypophysis of the recently mated rabbit. Hormones associated both with follicle maturation and with corpus luteum formation appear to be released from the female rabbit hypophysis through the stimulus of mating. Because this discharge continues for a period longer than that required for ovulation, it is probable that the gonadotropic hormones released become available for the subsequent production and proper development of corpora lutea. Fee and Parkes (2) and Smith and White (5) have shown that ovulation will occur if the hypophysis is removed one hour after mating, but that the subsequent development of corpora lutea under these conditions is abnormal.

Not only gonadotropic hormones but also thyrotropic and perhaps adrenal-stimulating hormones appear to be released from the female rabbit hypophysis during the period after mating. Evidence for the release of adrenal-stimulating hormones is, however, not conclusive, and more exact methods may be required to test this observation. Mating of the female rabbit appears to produce a discharge of several hypophyseal hormones. While it is not certain that this discharge is specific, many clinical and experimental studies have shown a relationship of the thyroids and adrenals to pregnancy. It is possible therefore that the discharge of hormones other than gonadotropic is also specific, and that stimulation of the organs on which these hormones act is necessary for the initiation and proper maintenance of pregnancy in this species. Krjlow and Sternberg (6) have shown that coitus causes in female rabbits a rapid and almost complete removal of colloid from the thyroid, with associated swelling of the acinar epithelium. During the middle period of pregnancy the colloid is again restored. It seems probable that the post-copulatory discharge of thyrotropic hormone is responsible for this stimulation. In a study of the hormone content of hypophyses from old sterile rabbits (4), it was found their hypophyses contained less thyrotropic and usually more adrenal-stimulating hormones than did those from young fertile females. A balance may be necessary for the proper initiation and maintenance of pregnancy.

SUMMARY

Changes in hormone content of the female rabbit hypophysis after mating were studied by implantation of hypophyses from recently mated rabbits into immature female guinea pigs. Glands obtained from rabbits 4 to 48 hours after mating produced less follicle maturation and less luteinization in the ovaries of the test animals than did those from normal unmated female rabbits of the same age. Glands obtained 24 and 28 hours after mating produced less stimulation of the thyroid as seen in microscopic section and less stimulation of the adrenal as measured by weight increase than did those from normal unmated female rabbits. These differences indicate that follicle-stimulating, luteinizing, thyrotropic and perhaps adrenal-stimulating hormones are released from the female rabbit hypophysis after mating.

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THE BIOLOGY AND CULTURE OF NEOAPLECTANA CHRESIMA, A NEW NEMATODE PARASITIC IN INSECTS¹

By R. W. GLASER, E. E. MCCOY, AND H. B. GIRTH

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A few years ago a number of corn earworms (*Heliothis armigera*) near Moorestown, New Jersey, were found dead and dying from a condition which on examination proved to be due to nematode parasitism.² Later the present writers found the same nematode parasitic in Japanese beetle (*Popillia japonica*) larvae. From 1937 to 1940 this parasite was found among the beetle larvae in 14 places distributed among 9 counties of New Jersey and in one locality in Maryland. In all these places this nematode seemed to represent the only helminth parasite present and was apparently causing a considerable reduction in the host population. Specimens of the nematode were submitted to G. Steiner of the Bureau of Plant Industry, United States Department of Agriculture, who stated that the form represented a new species of the genus *Neoapectana*. He will shortly publish a description of this and other new species and a key to all known forms of the genus *Neoapectana*. Steiner furnishes the following diagnosis which is published here with his permission, the name of the species being derived from the Greek word *χρήσιμα* meaning useful.

Neoapectana chresima Steiner

"*Diagnosis: Neoapectana* resembling *N. glaseri* Steiner, 1929 (1), but the adults of both sexes different by a sharply set off, often mucronate, tail terminus, by an excretory pore opening much closer cephalad, i.e., at a latitude near the middle of the corpus of the esophagus or at about one-third of the total length of the esophagus. Spicula proximally capitate and curved outward. Arrangement of male copulatory papillae somewhat different. Larvae with four equidistant longitudinal striae on

¹ A study conducted cooperatively by The Rockefeller Institute for Medical Research, Princeton, N. J., the New Jersey Department of Agriculture, and the United States Department of Agriculture.

² On November 26, 1934, Dr. W. H. Larrimer of the Bureau of Entomology and Plant Quarantine collected three diseased pupae of the corn earworm (*Heliothis armigera* Hbn.) from the soil in an outdoor hibernation cage at Moorestown, New Jersey. These were examined by Dr. Vera K. Charles of the Bureau of Plant Industry, who found them to contain a large number of nematodes and transmitted the material to Dr. G. Steiner of that Bureau for further examination. Steiner pronounced them to be representatives of a new species of the genus *Neoapectana*.

lateral fields, tail elongate-conical, terminus not set off, the phasmids located near middle.

"Type host: *Heliothis armigera* Hbn.

"Type locality: Moorestown, N. J., U. S. A."

Our studies showed the life cycle of *N. chresima* to be similar to that of *N. glaseri* (2). The adults of *N. chresima* are somewhat like *N. glaseri* but smaller. As in the latter species the females are ovoviviparous, but each produces between 250 and 400 young in contradistinction to *N. glaseri*, which generally produces approximately 15 young. (Certain rare "giant" females of *N. glaseri* may produce as many as 1400 young (3).) At first it was thought that *N. chresima* females were oviparous as well as ovoviviparous because free embryonated ova were often found. After careful study, however, it was concluded that while such free embryonated ova may develop normally they were derived from mature females that ruptured in a non-isotonic solution. In an isotonic solution free ova are not obtained, and *N. chresima* females then give birth only to living young. The larval forms are smaller than the corresponding stages in *N. glaseri*. The infective stage and the adults are more sluggish. The second-stage *N. chresima* larvae ensheath more rapidly than is the case with *N. glaseri*. Dead Japanese beetle larvae parasitized by *N. chresima* rarely smell badly, and when they do the parasites are usually dead. In general, the grub cadavers assume a dirty dull yellow color in contradistinction to an ochreous brown color of those parasitized by *N. glaseri*. In the former case the larval contents become quite viscid and of a dirty yellow hue. In the latter the contents are more fluid and of a clear brown color. When Japanese beetle larvae are experimentally exposed to soil or food contaminated with *N. chresima* they die in about 4 days.

Other hosts experimentally infected with *N. chresima* were the grasshopper (*Melanoplus* sp.), the European corn borer (*Pyrausta nubilalis*), the corn earworm (*Heliothis armigera*), and catalpa sphinx caterpillars (*Ceratomia catalpa*). In one of these experimentally infected catalpa sphinx caterpillars the nema reproduced to such an extent that at death 1,140,000 second-stage larvae were counted.

It was important to know whether *N. chresima* could be cultured on artificial media as is the case with *N. glaseri* (2). Occasionally *N. chresima* would show slight development in some particular medium, but it could not be carried through a second transplant. Such cultures often smelled badly, and it was assumed that bacteria were responsible for the failures. Second-stage larvae of *N. chresima* were then subjected to a sterilizing process described in 1940 (4, 5). After the nemas were bacteria-free they grew well on pieces of fresh, sterile rabbit kidney and survived in transplants.

The method was the following. A piece of kidney weighing one or two grams is placed in the water of condensation at the bottom of an ordinary nutrient agar slant after which the surface of the tissue is inoculated with

the nematodes. To prevent evaporation the cotton plug of the tube is trimmed and pushed down, and the surface covered with sealing wax. By means of a hot wire a small perforation is later made through the hardened wax. All tubes are held in a slanted position and incubated at room temperatures, 22–28°C. The nutrient agar is not a necessary nutritional constituent. Kidney alone suffices, but under the conditions described above the slanted agar affords a surface upon which the nematodes can migrate when they are impelled to do so. Some of these sterile cultures of *N. chresima* have been maintained for about two years and are to date in their 28th transplant. Transplants may be made at intervals of one to three months.

Recently an autoclaved medium has been devised which gives excellent growth and, because it can be more cheaply prepared in larger amounts, has replaced the older fresh rabbit tissue method. This medium is a semisolid gel at pH 7.0, in the proportion of 20 gm ground beef kidney or liver, 100 ml water, 0.5 gm sodium chloride, and 0.5 gm agar. This is autoclaved and may be stored for two weeks prior to use. It may be used for *N. glaseri* as well as for *N. chresima*, although it is not the substrate which is usually employed for the former species. Since both species are aerobic they develop on and near the surface of the semisolid gel. This gel offers enough resistance so that the nemas do not perish by sinking and further obtain enough foothold, so to speak, to effect their molts. Every two days the flasks containing the cultures are gently shaken to bring fresh nutrients from the lower layers to the surface. Under these conditions the cultures ordinarily reach stability in about 15 to 30 days, depending upon the amount of medium, the number of nemas initially used as inoculum, and fluctuations in the daily temperatures. When the cultures have reached stability no further increase in numbers is obtained on longer incubation and the dominant form is the infective larva.

A comparative count on the above medium for *N. glaseri* and *N. chresima* follows. Fifty ml of medium were used throughout, and the flasks were initially inoculated with approximately 10,000 infective forms of *N. glaseri* or *N. chresima* respectively. The final counts (recorded below as the average of 5 separate samples) were made in 23 days, at which time these cultures had reached stability. The cultures were free of all microorganisms, as comprehensive sterility tests showed.

Nutrient in medium	Final count for <i>N. glaseri</i>	Final count for <i>N. chresima</i>
Beef liver.....	538,000	522,000
Beef kidney.....	428,000	386,000
Beef muscle.....	18,000	6,000

The above test shows that the two species grew well on both liver and kidney but not at all on beef muscle. The counts on the last nutrient, within the experimental error, indicate no increase above the number initially introduced.

In 1940 (5) the importance of sterility was emphasized in rearing *N. glaseri* to obtain the maximum yield. As regards *N. chresima*, no growth is ordinarily obtained when contaminants are present. However, in the presence of one bacterium a number of strains of *N. chresima* have developed and have consistently yielded cultures on transplantation. This contaminant is a non-putrefactive, nonsporulating, gram negative, motile bacillus originally derived from the soil. Cultures of *N. chresima* associated with this seemingly innocuous bacillus are not so luxuriant as are the sterile cultures. The data below illustrate this point. The experiments were performed as previously described except that a smaller amount of medium and a smaller nema inoculum were used.

Final counts for *N. chresima* cultures
(Nutrient in medium is beef liver)

Strain No.	Sterile	Contaminated with one bacterial species
1.....	266,000	199,000
2.....	280,000	14,000
3.....	300,000	86,000

The sterile cultures of *N. chresima* have now been carried by transplantation for two years, and those contaminated with the one bacillus for one and one-half years. For future physiological studies, an attempt is now being made to adapt *N. chresima* to liquid nutrients such as the nutrient solution for *N. glaseri* described in 1940 (5).

No field tests, to appraise the economic possibilities of *N. chresima*, have as yet been attempted.

SUMMARY

A new nematode, preliminarily described by Steiner as a new species, *Neoaplectana chresima*, was found naturally parasitic in two species of insect hosts, and five additional species of insects were found to be susceptible to experimental infection. Certain biological differences between *N. chresima* and *N. glaseri* are briefly outlined. The sterile culture of *N. chresima* is described and the deleterious effects of contaminants emphasized.

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SEPARATION OF TOBACCO NECROSIS VIRUS AND TOBACCO MOSAIC VIRUS

By SEYMOUR S. COHEN*

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for
Medical Research, Princeton, New Jersey)

Introduction. During a study of the properties of tobacco necrosis virus, occasional batches of Turkish tobacco plants were encountered which developed not only the non-systemic tobacco necrosis on the lower inoculated leaves, but also through contamination the systemic tobacco mosaic disease. The yield of tobacco mosaic virus (TMV) may be between 2-3 mg per cc of juice,¹ or 100 times that of tobacco necrosis virus (TNV), the average yield of which is about 0.02 mg per cc.² Therefore, a single plant carrying a mixed infection in a batch of 100 plants diseased with tobacco necrosis will eventually yield a preparation of TNV containing approximately an equal amount of TMV. In order to avoid the complete loss of TNV in such contaminated preparations, it was considered desirable to attempt the removal of TMV.

TNV is completely soluble over the entire pH range in the absence of large amounts of salt. Since TMV is almost completely insoluble in the region of the isoelectric range, preparations containing very large amounts of TMV were first isoelectrically precipitated. Pirie, *et al.*,³ have demonstrated the absence of cross reaction between TNV and rabbit TMV antiserum. TMV antiserum may, therefore, be used in the quantitative removal of amounts of TMV within the limits of the titer and amount of available antiserum. TNV was finally separated from the lighter serum proteins by sedimentation in the ultracentrifuge.

Although isoelectric precipitation of TMV apparently removed considerable amounts of TNV from solution, the separation by means of antiserum appeared to entail no loss of TNV.

Experimental. Leaves of Turkish tobacco plants having large numbers of necrotic lesions characteristic of tobacco necrosis were harvested 5-7 days after inoculation. The leaves were frozen and stored at -14°C till used. The frozen leaves were ground and allowed to thaw. The juice was expressed

* Fellow in the Medical Sciences of the National Research Council.

¹ Stanley, W. M., *J. Biol. Chem.*, 1937, **121**, 205.

² Cohen, S. S., and Stanley, W. M., unpublished data.

³ Pirie, N. W., Smith, K. M., Spooner, E. T. C., and MacClement, W. D., *Parasitology*, 1938, **30**, 543.

at 4°C and a purified virus solution was obtained in the usual manner after at least 4 differential centrifugation cycles, involving a hundredfold reduction in volume. A cycle consisted of ultracentrifugation in the quantity ultra-centrifuge at 30,000 rpm for 75 minutes, emulsification of the pellet in water or buffer and centrifugation in an angle centrifuge for 30 minutes at 3000

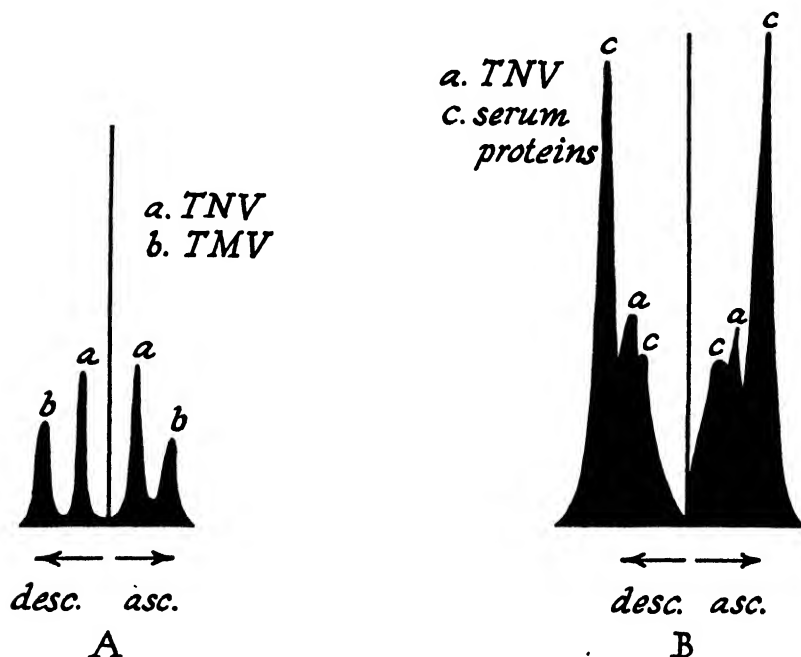


FIG. 1. Separation of tobacco necrosis virus (TNV) and tobacco mosaic virus (TMV) followed in Tiselius electrophoresis apparatus. A represents boundaries of mixture before separation. B represents boundaries after addition of antiserum to TMV. Boundaries were observed 1 hour after beginning of electrophoresis.

rpm. The supernatant was then ready for another cycle. All operations were carried out at about 4°C.

Preparations contaminated with TMV were found to have a much greater opalescence than those containing TNV alone. Such preparations were examined in an analytical ultracentrifuge^{4,5} equipped with a Svensson-Philpot⁶ optical system. In one case the virus solution before and after treatment with antiserum was examined at 0°C in the Tiselius electrophoresis apparatus

⁴ Bauer, J. H., and Pickels, E. G., *J. Exp. Med.*, 1937, **65**, 565.

⁵ Pickels, E. G., *Rev. Sci. Instruments*, 1938, **9**, 358.

⁶ Svensson, H., *Kolloid Z.*, 1939, **87**, 181.

equipped with the modification of the schlieren optical system described by Longworth.⁷

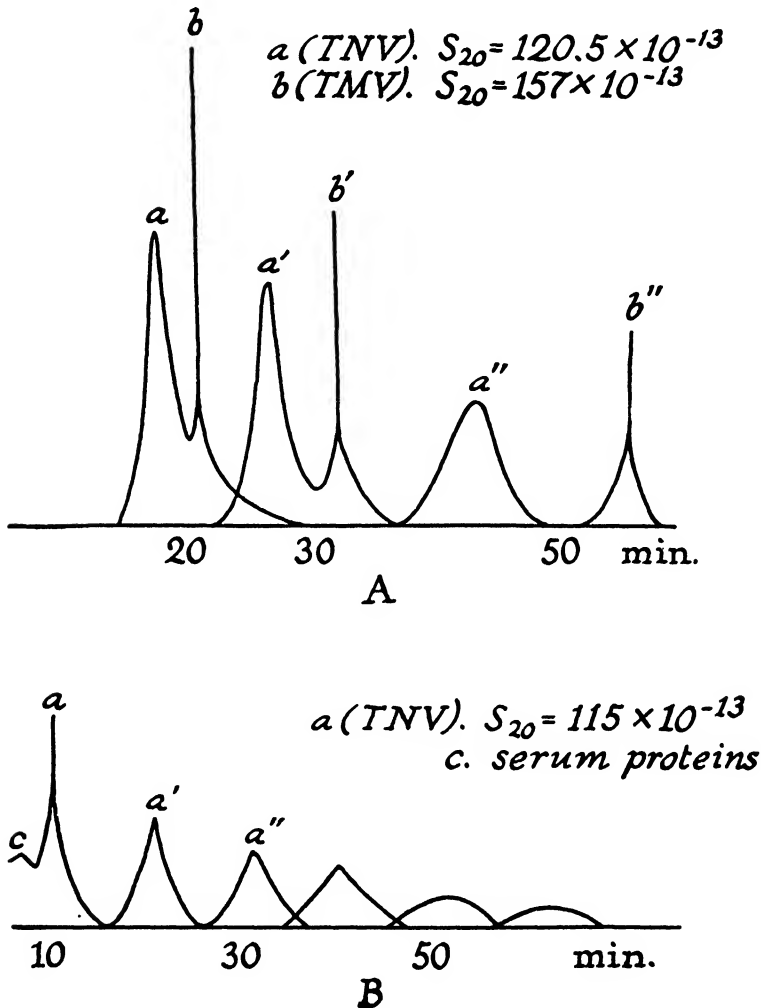


FIG. 2. Separation of tobacco necrosis virus and tobacco mosaic virus followed in analytical ultracentrifuge. A represents boundaries of mixture before separation. B shows separation of TNV from serum proteins after removal of TMV.

A virus solution containing 1.3 mg per cc TNV and approximately the same amount of TMV was subjected to electrophoresis at pH 6.72 in veronal buffer (0.02 N Na veronal and 0.08 N NaCl). The mobility of TNV (in $\text{cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$) was -1.85×10^{-5} ; that of TMV was -5.50×10^{-5} . The

⁷ Longworth, L. G., *J. A. C. S.*, 1939, **61**, 529.

current was reversed and the boundaries returned to their original positions. To 12 cc of the recovered virus mixture, 2 cc of rabbit TMV antiserum was added. After standing in the cold room overnight, the precipitate was centrifuged off and the solution dialyzed 24 hours against veronal buffer. The solution which was at pH 6.76 was again subjected to electrophoresis. The peak comparable to TNV possessed a mobility of -2.25×10^{-5} . After 3 hours no boundaries other than those of TNV and serum proteins could be detected. It is noteworthy that there is practically no loss of TNV as indicated by the size of the peaks. (Fig. 1.)

In a second experiment, TMV was first isoelectrically precipitated and small residual amounts subsequently removed with TMV antiserum. Fig. 2 represents the sedimentation of the virus boundaries before and after separation. In this case, the sedimentation constant of TMV was 157×10^{-13} cm/sec/unit field, indicating a concentration of approximately 10 mg/cc.⁸ To 10 cc of this virus solution, 0.2 N HCl was added till the pH was 3.5. The solution was placed in the icebox for 1 hour. The supernatant liquid after centrifugation at 3000 rpm for 30 minutes was adjusted to pH 7.4 with 0.2 N NaOH. This solution after addition of 0.5 cc TMV antiserum was placed in the icebox overnight. After centrifugation at 3000 rpm for 15 minutes, the supernatant liquid was examined in the analytical ultracentrifuge. The boundary due to TMV had been eliminated.

While the analytical ultracentrifuge and Tiselius apparatus are quite sensitive, it is impossible to detect by means of these instruments concentrations of TMV 100 times greater than is necessary to produce tobacco mosaic in Turkish tobacco. Therefore, the final test of the effectiveness of the separation resided in the determination of the biological activities of the preparations. Before separation, the preparations of mixtures at a concentration of 10^{-5} g/cc protein in 0.1 M phosphate at pH 7.1 caused both tobacco mosaic and tobacco necrosis to develop in Turkish tobacco. After the procedure described above, the same protein concentration produced only tobacco necrosis in Turkish tobacco plants.

Summary. Preparations of mixtures of TMV and TNV were obtained from plants having both diseases. These viruses were then separated by removal of TMV by isoelectric precipitation and by means of absorption with rabbit antiserum to TMV. The separation was followed by means of the analytical ultracentrifuge, the Tiselius electrophoresis apparatus and the biological activities of the preparations.

⁸ Lauffer, M. A., *J. Phys. Chem.*, 1940, **44**, 1137.

THE ACTION OF INTESTINAL NUCLEOPHOSPHATASE ON TOBACCO MOSAIC VIRUS

By SEYMOUR S. COHEN* AND W. M. STANLEY

*(From the Department of Animal and Plant Pathology of The Rockefeller
Institute for Medical Research, Princeton, New Jersey)*

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Tobacco mosaic virus is a nucleoprotein having a molecular weight of approximately 5×10^7 (1). The nucleic acid, which comprises slightly more than 5 per cent of the virus, is of the ribose type (2) and is bound to virus protein in a mode of linkage as yet unknown. Investigation of tobacco mosaic virus structure by means of x-ray analysis (3) and electron microscopy (4) has not indicated a concentration of the comparatively dense nucleic acid in any particular part of the nucleoprotein molecule. The nucleic acid may therefore be uniformly distributed in the virus molecule. The many methods which have been devised to remove nucleic acid from virus protein are, in general, methods which result in loss of virus activity and disruption of the virus particle into much smaller protein fragments. These denaturation procedures include treatment with urea (5), alkali (6), acid (7), pressure (8), and heat in the presence of detergents.¹ Heat treatment in the presence of buffer results in the denaturation of the protein and liberation of the nucleic acid (7).

Of considerable interest, therefore, is a recent paper by Schramm (9) that deals with the action on tobacco mosaic virus of an intestinal nucleophosphatase preparation, first described by Klein (10). Certain experimental data obtained by use of this enzyme under conditions suggested by Brederick and Müller (11) were interpreted by Schramm as indicating that the nucleic acid is completely removed from the nucleoprotein, leaving a protein whose size, homogeneity in the ultracentrifuge, and electrophoretic

* Fellow in the Medical Sciences of the National Research Council.

¹ Cohen, S. S., unpublished data.

mobility are the same as those of the original virus. The importance of this conclusion with respect to questions of virus and protein structure, as well as its uniqueness as compared to the results of other methods of investigation, required that an attempt be made to confirm this work.

The results obtained in the present investigation indicate that, under conditions approximating those described by Schramm, intestinal nucleophosphatase failed to remove nucleic acid from the virus. The activity of our enzyme preparation was established by its ability to hydrolyze both yeast and virus nucleic acid. An exact duplication of the experiments was made very difficult by the fact that the pH range stated to be necessary for the activity of the enzyme could not be attained under the conditions described. However, this pH range was achieved in other experiments. In addition, many variations of different parts of the experimental procedure, such as pH, time, age of virus preparation, etc., were made in attempts to secure conditions which might lead to the results described by Schramm. However, in no instance did exposure to the enzyme result in inactivation of the virus and separation of the nucleic acid.

EXPERIMENTAL

Preparation of Enzyme—Approximately 1 yard of intestine, immediately following the stomach, was removed from each of twenty freshly slaughtered calves. The intestines were cleansed with tap water and the intestinal mucosa scraped with a spatula. 925 cc. of 87 per cent glycerol were added to 186 gm. of mucosal brei and the mixture was shaken for 2 days at room temperature. 12 cc. of toluene were added and the enzyme preparation was stored at 4°. This procedure conforms in every detail to the modification of Klein's method as described by Schramm.

Activity of Enzyme on Yeast Nucleic Acid—It was considered necessary initially to establish the activity of the enzyme preparation towards a substrate described by Klein. Accordingly, the enzyme was tested on yeast nucleic acid (Merck) at three different hydrogen ion concentrations. The yeast nucleic acid was dissolved in 0.1 N NaOH to give a pH of 7.0 and the solution was filtered and used without further purification. The solution contained 1.70 mg. of P per cc. To 10.0 cc. of this substrate

were added 0.2 cc. of toluene, 2.7 cc. of 0.5 N veronal buffer of the desired pH, and 2.0 cc. of the enzyme solution. In the control experiments, water replaced the enzyme solution. The mixtures were incubated at 37° for 72 hours. At the start and at the end of the incubation period, 2.0 cc. samples were removed for pH determinations. These were measured by means of a glass electrode and the results are recorded in Table I.

To 10 cc. of each reaction mixture, 15.0 cc. of 10 per cent trichloroacetic acid were added at room temperature, and the mixture was kept at 4° for 1½ hours. The solution was filtered, and 0.5 cc. of 95 per cent alcohol were added to 9.5 cc. of filtrate. The mixture was kept at 4° for 24 hours. The supernatant liquid

TABLE I
Hydrolysis of Yeast Nucleic Acid (YNA) by Nucleophosphatase at Different Hydrogen Ion Concentrations

Preparation	pH at start	pH at end	Free P	Total free P in reaction mixture	Amount of hydrolysis
			<i>mg. per cc.</i>	<i>mg.</i>	<i>per cent</i>
YNA.....	7.0	6.9	0.46	17.8	100
Control.....	7.0	7.2	0.19	7.4	
YNA.. . . .	8.0	7.7	0.45	17.5	100
Control.....	8.0	8.1	0.17	6.6	
YNA.. . . .	9.0	8.1	0.46	17.8	100
Control.....	9.0	8.8	0.20	7.8	

obtained after centrifugation at 3000 R.P.M. for 15 minutes was examined for phosphorus by the King method (12). The results of these analyses are given in the fourth column of Table I. By the same method of analysis, 2 cc. of enzyme alone yielded 0.54 mg. of P and 10 cc. of the solution of yeast nucleic acid yielded 6.61 mg. of P. These figures correspond to the total of free and soluble phosphorus of the preparations under the conditions of the analytical method. From Table I, which summarizes the results, it can be seen that very little additional free phosphorus appeared in the control experiments, while all the phosphorus appeared in the free form in the system containing the enzyme. The experiments show that the enzyme preparation completely hydrolyzed yeast nucleic acid over the pH range of 7 to 9.

Activity of Enzyme on Virus Nucleic Acid—The preparation of virus nucleic acid used in the present studies was isolated by Dr. H. S. Loring by means of alkaline denaturation of tobacco mosaic virus (2) and was subsequently purified by dialysis. We have subjected this preparation of virus nucleic acid to ultracentrifugation, diffusion, and electrophoresis studies and found it to be quite homogeneous with a molecular weight of 11,000.¹ A description of these experiments will be published later in greater detail. A sample of this material was incubated with enzyme at pH 7.5 in 0.1 M veronal buffer for 96 hours. The experiment was carried out in the manner described previously for yeast nucleic acid. The results indicated a 96 per cent hydrolysis of this material by the enzyme preparation.

Action of Enzyme on Tobacco Mosaic Virus—The enzyme preparation was next tested on tobacco mosaic virus (TMV) under conditions exactly comparable to those described for the yeast nucleic acid. The virus solution was prepared from virus freshly isolated by differential centrifugation and contained 18.9 mg. per cc.

At the start and at the end of the incubation period, samples were removed for determination of pH. The solution was centrifuged for 20 minutes in an angle centrifuge at 3000 R.P.M. The slightly turbid supernatant fluid was decanted, and 10.0 cc. of this solution were sedimented in the quantity ultracentrifuge. This procedure was undertaken to recover all substances having a size comparable to that of tobacco mosaic virus, since the method of analysis for bound phosphorus described by Schramm was considered to be unsatisfactory.² The pellet obtained on ultracentrifugation was dissolved in water and the volume of the solution was adjusted to 10.0 cc. The solution was dialyzed overnight at 4° against distilled water and centrifuged at 3000 R.P.M. for 15 minutes. The supernatant liquid was used for determinations of

² It was found on attempting to use Schramm's procedure that the addition at room temperature (22°) of 30 per cent trichloroacetic acid to twice the volume of virus solution caused a liberation of 20 per cent of the phosphorus present in enzyme-treated and untreated virus preparations. The elevation of the temperature to 100° after addition of the trichloroacetic acid caused practically quantitative removal of the phosphorus from both types of protein preparations.

nitrogen, phosphorus, and biological activity. Nitrogen was determined by the Kjeldahl method. This value multiplied by the factor 6.25 was used to estimate the protein content of the preparation.

Biological activity was determined by the half leaf method (13) on *Phaseolus vulgaris* L., and a minimum of thirty-one leaves was used in each test. The enzyme-treated virus was compared to virus undergoing the control experiment. The virus concentrations used were approximately 2×10^{-5} gm. per cc.

The results, which are summarized in Table II, clearly show that, following exposure of tobacco mosaic virus to the nucleo-

TABLE II
Action of Nucleophosphatase on Tobacco Mosaic Virus (TMV) in Buffered Systems

Preparation	pH at start	pH at end	Virus recovered	Bound P	Virus P	Virus activity
			per cent	mg. per cc.	per cent	per cent
TMV..	7.0	7.3	89	0.067	0.58	117
Control.. . .	7.0	6.9	96	0.071	0.58	
TMV	8.0	7.9	95	0.061	0.50	97
Control.. . .	8.0	7.9	93	0.064	0.54	
TMV*.. . . .	9.0	8.5	81	0.055	0.53	76
Control*.	9.0	8.7	66	0.046	0.54	

* The fact that tobacco mosaic virus is unstable in the vicinity of pH 9 probably accounts for the low yields and decreased activity of the nucleoprotein recovered after incubation at that pH.

phosphatase, practically all of the virus may be recovered and that the virus so recovered possesses the normal activity and phosphorus content. The experiments demonstrate that the virus is unaffected by conditions known to bring about the hydrolysis of free nucleic acid. It may be noted that ribonuclease brings about the hydrolysis of free virus nucleic acid but, although causing the reversible inactivation of tobacco mosaic virus, has no effect on the nucleic acid when bound to protein in the form of virus (14).

Activity of Enzyme on Tobacco Mosaic Virus in System without Added Buffer—In view of the results described above, it appeared desirable to repeat the experiments in the absence of added buffer in an attempt to approximate more nearly the conditions described

by Schramm. Two different virus preparations were used in these experiments. One containing 17.31 mg. per cc. was obtained by differential centrifugation; the second, containing 11.05 mg. per cc., was considerably older and had been purified by both $(\text{NH}_4)_2\text{SO}_4$ precipitation and differential centrifugation. The former, designated as TMV, was found to contain 0.49 per cent phosphorus, and the latter, designated as TMV-A, was found to contain 0.50 per cent phosphorus.

A series of four tubes was set up with each virus preparation. To 15.0 cc. of virus solution were added 2.0 cc. of the enzyme solution, 0.2 cc. of toluene, 4 drops of 0.1 per cent phenolphthalein, and sufficient 0.2 N NaOH (between 0.2 and 0.3 cc.) to bring the system to the phenolphthalein end-point. In some cases the end-points were deliberately exceeded in the hope that some variation in pH might be sufficient to produce the effects claimed by Schramm. However, the actual pH of all tubes was determined by means of a glass electrode before incubation for 72 hours at 37°, and one tube from each series was used to follow the hydrogen ion concentration of the system during the incubation period. After incubation the solutions were titrated with 0.02 N NaOH back to the phenolphthalein end-point. Within the period of incubation, the pH of the solutions apparently dropped from about 8.9 to about 8.3, since actual determinations by means of a glass electrode showed that TMV-1 dropped from pH 8.95 to 8.31 and TMV-A-3 from pH 8.89 to 8.32. Under these conditions, according to Schramm, the pH of the system should have fallen to between pH 7 and 8.

At the conclusion of the titration, all material possessing a size comparable to that of tobacco mosaic virus was isolated as described in the preceding experiment and analyzed for nitrogen and phosphorus. The results, summarized in Table III, clearly show that nucleic acid was not removed in significant amounts from either fresh or old virus nucleoprotein.

Four additional experiments carried out under the same conditions just described, except that an incubation period of 144 hours was used, showed substantially the same results. The percentage of phosphorus in the recovered sedimentable material was 0.54 for TMV and 0.50 for TMV-A.

Since under the conditions described by Schramm the pH range

of the reaction mixtures in the experiments described above apparently did not reach that which had been used by Brederick and Müller, *i.e.* pH 7 to 8, a further test of enzymatic activity was made in the pH range 7.3 to 7.5 in the absence of added buffer. Two groups consisting of two tubes each were set up with TMV (18.9 mg. per cc.) and TMV-A (11.05 mg. per cc.). To 10.0 cc.

TABLE III

Activity of Nucleophosphatase on Tobacco Mosaic Virus over pH Range 8 to 9 in Absence of Added Buffer

Preparation	pH at start	Amount of 0.02 N NaOH added	pH at end	Virus recovered	Bound P	Virus P
		cc.		per cent	mg. per cc.	per cent
TMV-2	8.9	2.30	9.1	76	0.054	0.48
3	8.9	1.95	9.0	81	0.055	0.46
4	8.9	1.30	8.8	83	0.055	0.45
TMV-A-1	8.8	1.40	8.8	73	0.030	0.43
2	8.8	1.55	8.8	72	0.029	0.42
4	9.2	1.05	8.7	61	0.025	0.43

TABLE IV

Activity of Nucleophosphatase on Tobacco Mosaic Virus at pH 7.5

Preparation	pH at start	Amount of 0.02 N NaOH added	pH at end	Virus recovered	Bound P	Virus P
		cc.		per cent	mg. per cc.	per cent
TMV-1	7.50	0.20	7.36	75	0.047	0.46
2		0.30		81	0.061	0.46
TMV-A-1	7.50	0.35	7.30	69	0.022	0.55
2		0.68		64	0.036	0.53

of substrate were added 2.0 cc. of enzyme, 0.2 cc. of toluene, and 3 drops of 0.1 per cent brom-thymol blue. The mixture was then adjusted to the blue-green end-point with measured small amounts of 0.2 N HCl. A portion of one tube of each group was used to follow the pH variation of the system. The mixtures were incubated for 72 hours at 37°.

At the conclusion of the incubation period, sedimentable material was isolated and analyzed as described previously. The

results, summarized in Table IV, again demonstrate that the enzyme failed to remove nucleic acid from the virus nucleoprotein in the pH range of 7 to 8.

SUMMARY

An enzyme preparation obtained from the intestinal mucosa of calves by the modification of Klein's method as described by Schramm has been found to hydrolyze yeast and virus nucleic acid. However, the enzyme was found to have no effect on tobacco mosaic virus, since, following incubation with the enzyme under a variety of conditions, virus possessing the normal infectivity and phosphorus content was always recovered in good yields. The report of Schramm that the enzyme effects the complete removal of nucleic acid from the virus and yields an inactive phosphorus-free protein which is the same as the intact virus with respect to size, homogeneity in the ultracentrifuge, and electrophoretic mobility has not been confirmed.

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DERIVATIVES OF TOBACCO MOSAIC VIRUS

I. ACETYL AND PHENYLUREIDO VIRUS*

By GAIL LORENZ MILLER AND W. M. STANLEY

(From the Department of Animal and Plant Pathology of The Rockefeller
Institute for Medical Research, Princeton, New Jersey)

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An investigation on the relationship of chemical structure to biological activity possesses a twofold interest in the case of viruses. First of all, one may assay the physiological importance of various functional groups in the molecule by converting these groups into chemically inactive forms and testing the resulting derivatives for infectivity. In addition, when a given derivative is found to be biologically active, one may determine the nature of the virus which is produced in the cells of the host. If the infecting molecules served as exact models for reproduction, one would expect to reisolate the virus derivatives from plants so infected. Furthermore, the disease produced by such an altered virus might be different from the normal disease, since it is known that the nature of a virus disease varies with the strain of the virus and that strains of a virus differ from one another in their chemical properties (1, 2). It is possible, therefore, that one might cause structural changes *in vitro* which would, in effect, correspond to the mutation of a virus. If, on the other hand, the inoculation of the virus derivative resulted in the production of normal virus, it might be concluded that the structural changes were reversed within the cells of the host or that that portion of the molecules involved in the structural change was unimportant and played a subordinate rôle in the reactions of virus reproduction.

It has been shown that the sulfhydryl groups of tobacco mosaic virus can be oxidized with iodine without changing the specific virus activity, but that inoculation of the oxidized virus is followed

* A preliminary report of the present work has been given (*Science*, **93**, 428 (1941)).

by the production of normal virus (3). Schramm and Müller (4) have reported that the amino groups of tobacco mosaic virus could be completely covered by treatment with ketene or phenyl isocyanate without a decrease in specific virus activity, but it was not determined whether the derivatives were propagated as such in susceptible hosts. Prolonged treatment with ketene resulted in inactivation of the virus. This was suggested to be due to involvement of tyrosine phenolic groups, although no chemical proof was given. In the present study, which was begun before the above work came to our notice, tobacco mosaic virus was acetylated and results in qualitative agreement with those of Schramm and Müller were obtained, although from a quantitative standpoint certain differences were observed. We have therefore extended the investigation, with particular regard to determining the nature of the virus which is propagated in plants which have been infected with derivatives of the virus.

In the first experiments, samples of tobacco mosaic virus in 1 M acetate buffer at pH 5.5 were treated with ketene for varying periods of time up to 4 hours. After each period of acetylation, the virus solutions were dialyzed against distilled water at 4° and the preparations thus obtained were used for chemical and biological tests. The changes in amino nitrogen were followed by the ninhydrin method as applied by Ross and Stanley (5). The decrease in tyrosine plus tryptophane groups was determined with Folin's phenol reagent by Herriott's method (6). Tests for biological activity were carried out on plants of both *Nicotiana glutinosa* and *Phaseolus vulgaris* by the half leaf method as used in this laboratory (7). The results are presented in Table I. In Experiment 1 it may be seen that the amino groups were covered more rapidly than the tyrosine plus tryptophane groups and seemed to reach a more or less constant figure representing 40 to 50 per cent coverage. No significant decrease in virus activity was detectable in any of these preparations. In Experiment 2, in which a more rapid flow of ketene was maintained, the total final coverage of amino groups was not appreciably increased, although an increase in rate of acetylation of tyrosine plus tryptophane groups was manifested. The extent of acetylation of these latter groups, like the former, now appeared to approach a limiting plateau, which in this case corresponded to about 20 per cent

acetylation. The partial inactivation evident in the 120 minute run was apparently due to secondary reactions, since the extent of acetylation of the groups tested was not affected. When a very rapid stream of ketene was employed, as indicated in Experiment 3, the extent of acetylation still could not be forced much beyond 50 per cent coverage of amino groups and 20 per cent coverage of the phenol plus indole groups. The virus derivative resulting from a 30 minute treatment under these vigorous conditions was quite gelatinous in character and when examined in the ultracentrifuge was found to be considerably aggregated. However, since the material was found to possess normal virus

TABLE I
Acetylation of Tobacco Mosaic Virus in Acetate Buffer

Experiment No.	Rate of flow of ketene	Time of acetylation	pH at end (5.5 at start)	NH ₂ groups covered	Phenol + indole groups covered	Loss in activity
		<i>min.</i>		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	Slow	15	5.5	39	9	0
		30	5.5	46	7	0
		60	5.5	36	12	0
		240	5.0	54	18	0
2	Moderate	10	5.5	53	13	0
		30	5.4	44	19	0
		60	5.3	48	22	0
		120	4.5	45	19	40
3	Rapid	30	4.8	55	21	0
		120	4.5			

activity, it is probable that on dilution for biological assay the aggregated particles were dissociated. The 120 minute period of treatment with the rapid stream of ketene resulted in the conversion of the virus into an insoluble precipitate.

It seemed possible that more complete acetylation of the virus might be obtained if the reactions were carried out at a higher pH. 0.5 M phosphate buffer at pH 8.1 was chosen as a suitable medium in which to carry out such experiments. As shown in Experiment 1 of Table II, 30 minutes treatment with a rapid stream of ketene at the more alkaline reaction gave a preparation of virus only slightly more acetylated than the corresponding preparation in acetate buffer. In physical properties, however, the derivative

thus obtained showed much less tendency towards gel structure formation, and when the treatment with ketene was extended to 120 minutes no precipitation occurred, as was the case in acetate buffer under similar conditions. With the longer treatment, the groups tested were not further acetylated, although the virus was partially inactivated.

Experiments were next carried out in which larger samples of virus were treated with ketene for separate 1 hour intervals. After each period of acetylation, the virus was dialyzed, isolated by ultracentrifugation, and dissolved in phosphate buffer at pH 8.1 for further acetylation. The pH of the phosphate buffer remained above 7 for the first half hour of each period of reaction. By acetylating for the separate periods, therefore, the virus was

TABLE II
Acetylation of Tobacco Mosaic Virus in Phosphate Buffer

Experiment No.	Conditions	Time of acetylation	pH		NH ₂ groups covered		Phenol + indole groups covered	Loss in activity
			Start	End	Nin-hydrin	Van Slyke		
					per cent	per cent	per cent	per cent
1	Rapid ketene	30	8.1	6.3	58		21	0
	Small scale	120	8.0	4.5	59		20	40
2	Rapid ketene	60	8.1	5.4	46	64	19	0
	Large scale	120	8.1	5.2	62	75	23	25
		240	8.1	5.2	75	83	22	50

treated for longer periods of time at an alkaline pH. For these experiments, the coverage of amino groups was determined by the Van Slyke nitrous acid method (8) as well as by the ninhydrin method. As may be seen from the data for Experiment 2, Table II, the slight further acetylation obtainable under these conditions was accompanied by some inactivation. The per cent coverage of amino groups as determined by the Van Slyke method was found to be somewhat higher than that obtained by the colorimetric method, indicating some lack of specificity in the latter. Thus, values of 55 to 58 per cent, which represent the maximum coverage without inactivation as determined by the ninhydrin method, correspond to a figure of around 70 per cent coverage as determined by the Van Slyke procedure. It may therefore be concluded

from the data as a whole that not more than 70 per cent of the amino groups of tobacco mosaic virus may be substituted without a concomitant loss of specific virus activity. With regard to the phenol plus indole groups, it may be concluded that at least 20 per cent and possibly more of these groups may be covered without the loss of infectivity of the virus.

The amino nitrogen of nine different samples of untreated virus, as determined by the Van Slyke method, was 0.13 ± 0.01 per cent. The analyses were carried out at 24° and digestion with nitrous acid allowed to proceed for 20 minutes. Under similar conditions, Schramm and Müller (4) obtained the value of 0.25 per cent amino nitrogen. In addition, they reported negative ninhydrin and Van Slyke tests for their acetylated virus. Since the authors stated that their virus stock was obtained from this laboratory, it is unlikely that the discrepancies can be explained on the basis of different strains of virus.

In order to determine whether tyrosine or tryptophane or possibly both of these amino acids were being affected by the ketene, analyses with the Folin phenol reagent were also made by the "pH 11 method" of Herriott (6). Herriott found that the chromogenic power of acetylated tyrosine derivatives could be recovered by treatment with alkali at pH 11, whereas that of acetyl tryptophane could not. We have found that the acetylated virus after treatment with NaOH at pH 11 gave with the Folin reagent 97 per cent of the color given by a control of unacetylated virus. It therefore seems probable that a portion of the tyrosine in the virus, but little or none of the tryptophane, was affected by the acetylation. The calculation of the exact extent of acetylation of tyrosine in the molecule was made rather uncertain, however, by the fact that the unacetylated, unhydrolyzed virus was found to yield only 59 per cent of the color produced by an equivalent amount of virus following complete hydrolysis by means of 6 N NaOH.

The amount of acetylation of the virus was also checked by acetic acid determinations on preparations of the derivative. Samples of normal and acetylated virus were hydrolyzed with NaOH according to the directions of Sandor and Tabone (9), the hydrolysates were acidified with an excess of citric acid, and the liberated acetic acid was separated by repeated vacuum distilla-

tion. The procedure was similar to that of Herriott (6) but was carried out on a smaller scale. 100 mg. samples of untreated virus liberated 0.0211 mm of acid, whereas the same sized samples of acetylated virus liberated 0.0323 mm of acid. The difference, or 0.0112 mm of acetic acid, was sufficient to account for the acetylation of 70 per cent of the amino groups of the virus and 20 per cent of the tyrosine phenol groups (10). It cannot be doubted, therefore, that the changes in amino nitrogen and in groups reactive to the phenol reagent which were observed on treatment of the virus with ketene were actually due to the acetylation of these groupings. The data indicate that even native virus may contain some acetyl groupings, although this has not yet been definitely established.

A preparation of acetylated virus in which the amino groups were covered to the extent of 70 per cent and tyrosine plus tryptophane groups to the extent of 20 per cent was inoculated at a dilution of 10^{-5} gm. of protein per cc. into a number of young Turkish tobacco plants. The disease produced in these plants was indistinguishable from that in a group of control plants. After a period of 4 to 5 weeks, the viruses produced in the test and control plants were isolated by differential centrifugation. The yields in the two cases were comparable. The virus isolated from the plants inoculated with the acetylated virus possessed the normal amino nitrogen content and showed the same chromogenic power towards the Folin reagent as did the virus from plants infected with normal virus. Further evidence was thus obtained that infecting virus molecules may not necessarily function as exact patterns for reproduction. However, as in the case of the iodine-oxidized virus (3), the objection might still be raised that the plant cells had transformed the derivative into the normal form before reproduction occurred.

In an effort to obtain a virus derivative less likely to be affected by the plant cells, samples of virus in phosphate buffer at pH 8 were treated with an excess of phenyl isocyanate to yield preparations of phenylureido virus. The data obtained with these derivatives are summarized in Table III. It may be seen that with sufficient time of reaction approximately the same per cent of amino groups was covered in this case as in the acetylation reactions. The coverage of phenolic groups was considerably greater after the

phenyl isocyanate treatment, but as with ketene the indole groups were found to be unaffected. No significant inactivation resulted from the conversion of normal virus to the phenylureido derivative. Furthermore, the disease which this derivative produced in Turkish tobacco plants was indistinguishable from that caused by ordinary virus, and the virus reisolated from the infected plants possessed the amino nitrogen content of untreated virus. When one considers the high stability of the phenylureido linkage at neutral and acid reactions (11) and the relatively foreign nature of such a linkage as compared with acetyl linkages in regard to occurrence in living tissues, one is inclined to discount the possibility that phenylureido virus molecules are hydrolyzed by cells of the host plants. If this conclusion is correct, it would follow that the acetyl virus as well was not necessarily hydrolyzed by

TABLE III
Phenylureido Tobacco Mosaic Virus

Experiment No.	Time	NH ₂ groups covered		Phenol + indole groups covered	Loss in activity
		Ninhydrin	Van Slyke		
	min.	per cent	per cent	per cent	per cent
1	90	25	43	43	0
2	410	50	63	36	0

the plant before its biological activity was exhibited. On the basis of the molecular weight of tobacco mosaic virus (12), it may be calculated that around 3000 amino groups and 2000 to 4000 phenolic groups per virus molecule were covered without loss of infectivity of the virus.

In order to determine whether the treated preparations were chemically uniform or consisted of molecules altered to widely different degrees, tests were made with the ultracentrifuge and the Tiselius electrophoresis apparatus. The homogeneity of the preparations as determined in the ultracentrifuge was not measurably altered by the two types of chemical treatment. However, because of the nature of the chemical changes involved, a more sensitive test was provided by the electrophoretic mobility. The electrophoresis experiments were carried out at pH 7.3 in 0.1 ionic K_2HPO_4 - KH_2PO_4 -KCl buffer in which 80 per cent of the

ionic strength was provided by the KCl. Runs were made on the normal virus, on the derivatives, and on mixtures of normal virus with each of the derivatives. Tracings of the Longworth

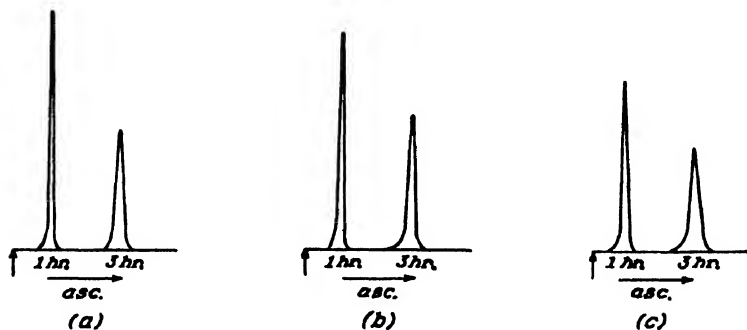


FIG. 1. Tracings of Longworth scanning diagrams of ascending boundaries obtained during electrophoresis of 0.1 per cent tobacco mosaic virus preparations. Field strength, 1.6 volts per cm. The vertical arrows indicate starting positions of boundaries. (a) Acetyl virus; (b) phenylureido virus; (c) normal virus.

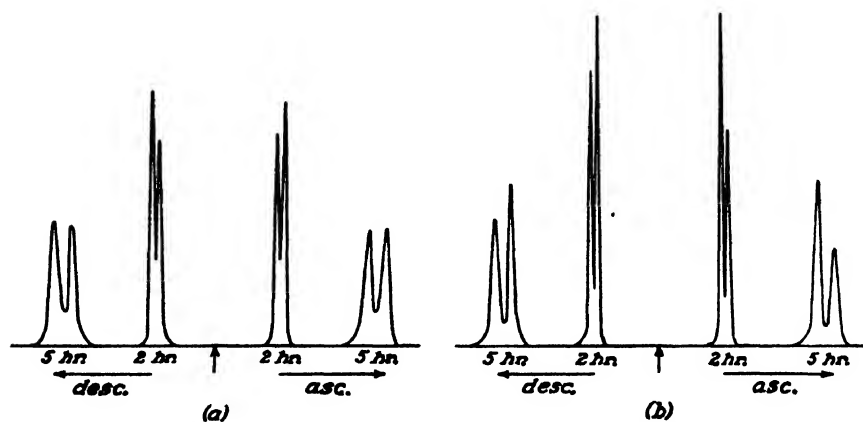


FIG. 2. Tracings of Longworth scanning diagrams of ascending and descending boundaries obtained during electrophoresis of mixtures of 0.1 per cent virus derivatives with 0.1 per cent normal virus. Field strength, 1.6 volts per cm. The vertical arrows indicate starting positions of boundaries. (a) Acetyl and normal virus; (b) phenylureido and normal virus.

scanning diagrams (13) for the various experiments are shown in Figs. 1 and 2. The normal virus possessed a mobility of -8.3×10^{-5} cm.² per volt sec., whereas the derivatives each possessed a

mobility close to -9.3×10^{-5} cm.² per volt sec. It may be seen from the diagrams that the derivatives were quite homogeneous with respect to electrochemical properties and that they could be separated readily from the normal virus when the latter was present. No appreciable amount of unchanged virus was evident in the preparations of the derivatives. The characteristic skewness observed in the scanning curves in certain cases is not indicative of electrochemical inhomogeneity but is the result of a slight streaming of the virus, similar in effect to that observed with vaccinia virus (14).

From the fact that preparations consisting of chemically uniform derivatives of tobacco mosaic virus possessed full biological activity, it must necessarily be concluded that individual units of the derivatives were infective. However, with regard to the question of the propagation of normal virus in plants infected with the derivatives, it might be argued that a very small amount of unchanged virus present in preparations of the derivative could, by virtue of a greater rate of movement or of reproduction within the plant, be solely responsible for the production of normal virus. This possibility was tested by a method based on the findings of Jensen (15) and of Kunkel (16) that, when plants giving the local lesion response were inoculated with a mixture of viruses, any single lesion which was isolated was usually found to contain only one of the viruses inoculated and not a mixture of viruses. Samples of the acetyl derivative of tobacco mosaic virus at concentrations of 10^{-6} to 10^{-8} gm. per cc. were rubbed on leaves of *Nicotiana glutinosa*. Ten single lesions from the infected plants were then removed and each was employed for the inoculation of a batch of young Turkish tobacco plants. For a control, a number of Turkish tobacco plants were simultaneously infected with stock normal virus. After the several inocula were allowed to increase for several weeks, the plants were harvested and the viruses were isolated by the usual procedure. The purified preparations were tested for amino nitrogen by the ninhydrin method and compared with control samples of normal virus. In no case were significant differences observed, and the amino nitrogen in all instances corresponded to that of normal virus. An experiment similar to the one just described was also carried out with the phenylureido virus and identical results were obtained. It has thus been es-

established that the normal virus propagated in plants infected with virus derivatives did not arise from normal virus present as a contaminant in the inoculum, and it may be concluded, therefore, that a large portion of the amino and phenol groups of the virus molecule may be altered without interfering with the basic reactions of virus reproduction.

It is of interest to compare the behavior of the above derivatives of tobacco mosaic virus with the behavior of similar derivatives of other well defined, biologically active proteins. Herriott and Northrop (17) found that with pepsin practically all of the amino groups could be acetylated by means of ketene without the loss of enzymatic activity, but that the progressive coverage of phenolic groups was accompanied by inactivation. Closely parallel results have been reported for insulin (18) and for human chorionic gonadotropin (19). On the other hand, diphtheria toxin (20), the pituitary follicle cell-stimulating, interstitial cell-stimulating, and lactogenic hormones (19, 21), and the gonadotropic hormone in pregnant mare serum (19) appear to require free amino groups for their activity. Gaunt and Wormall (22) found that the treatment of insulin with phenyl isocyanate led to the inactivation of the hormone. In control experiments it was found that the phenolic group of the free amino acid, tyrosine, did not react appreciably with phenyl isocyanate, and it was therefore assumed that the phenol groups of the insulin likewise were unaffected by the reagent. Our findings with tobacco mosaic virus have indicated a marked reaction between phenyl isocyanate and the phenolic groups in the virus protein. In view of the results with the virus and the fact that the acetylamino insulin is biologically active whereas the acetylamino, acetylphenolic insulin is inactive (18), it seems possible that the inactivation of the hormone by the phenyl isocyanate was due to the actual blocking of phenol groups in the molecule.

EXPERIMENTAL

Preparation of Acetyl Virus—Stock samples of tobacco mosaic virus were obtained by differential centrifugation of the juice expressed from diseased Turkish tobacco plants (23). After the virus was recentrifuged two or three times, it possessed an amino nitrogen content of 0.13 ± 0.01 per cent and was suitable for

acetylation experiments. In the first experiments ketene was passed through 20 cc. of a solution of 0.5 per cent virus in 1 M acetate buffer at pH 5.5. During the acetylation the virus was kept in a cellophane dialysis bag suspended in 1 liter of outside buffer at pH 7.5. A stream of ketene was introduced through a sintered glass filter. The ketene generator and trap for ketene polymers were adapted from the directions of Herriott and Northrop (17) and of Li (24). The acetylations were carried out for periods up to 4 hours. An effort was made to keep the solutions more alkaline than pH 4.8, because when they became too acid the virus was precipitated and inactivated. The temperature was 24°. The virus solutions at the end of reaction were dialyzed at 4° in a rocking apparatus (25) against 40 liters of distilled water. The large scale acetylations were carried out on 180 cc. portions of 0.5 per cent tobacco mosaic virus in 0.5 M potassium phosphate buffer at pH 8.1. In these cases, an outside buffer of 2 liters of 1 M K_2HPO_4 at pH 8.8 was used and was stirred mechanically during the runs. After each 1 hour period of acetylation, during which the pH dropped to 5.4 or 5.2, the virus was dialyzed free of phosphate, isolated by ultracentrifugation, and dissolved in phosphate buffer at pH 8.1 for further acetylation. The hydrogen ion concentrations were determined by means of a glass electrode.

Preparation of Phenylureido Virus—In a typical experiment, 100 cc. of 0.1 M phosphate buffer at pH 8.6 and containing 1.63 gm. of ultracentrifugally purified tobacco mosaic virus were cooled by means of an ice bath, and a total of 1.6 cc. of phenyl isocyanate and 7 cc. of 0.2 N NaOH was added with continuous stirring during the course of 2½ hours. The phenyl isocyanate was added to the cooled solution in 0.1 cc. portions at 10 minute intervals. The hydrogen ion concentration of the solution was maintained between pH 7.9 and 8.5 by the occasional addition of the alkali in 1 cc. portions. The preparation was then allowed to stand at 4° for an additional 260 minutes with occasional stirring. At the end of this time the hydrogen ion concentration was pH 7.5. The precipitated diphenylurea was removed by centrifugation and washed with 80 cc. of 0.1 M phosphate buffer at pH 7. The supernatant liquid, which was similar in appearance to solutions of ordinary tobacco mosaic virus, and the 80 cc. of wash liquid were combined and dialyzed in a rocking apparatus against 40 liters

of cold distilled water during the course of 24 hours. The final preparation contained 1.2 gm. of protein, corresponding to a yield of about 74 per cent based on the starting material.

Ninhydrin Amino Nitrogen Determinations—The ninhydrin tests were first carried out by the quantitative procedure used by Ross and Stanley (5). According to this method, 5 to 10 mg. samples of virus in 1 cc. of solution are mixed with 0.3 cc. of pyridine and 0.3 cc. of 2 per cent aqueous ninhydrin and allowed to stand for about 20 hours at 37° for color development to take place. Because of the variability encountered, it was essential to run each determination in triplicate. We have now found that by carrying out the reactions at 65–75° for a period of 30 minutes a much more rapid color development occurs and the variability is greatly reduced. The colored solutions thus obtained were diluted to 8 cc. and compared in the Klett-Summerson photoelectric colorimeter. A green filter No. 54 was used in the estimations. The results of determinations on acetylated virus preparations by the original and by the modified procedures were identical, showing that the heat treatment did not cause further liberation of amino groups. In the absence of pyridine, precipitation of the virus interfered with quantitative estimation. It was found that samples of untreated virus containing less than 2 mg. may yield no color with the ninhydrin reagent, and furthermore that even larger samples of the acetylated virus may give negative results. It was therefore important that sufficiently large samples be employed for the determination. The negative ninhydrin tests reported by Schramm and Müller may have been due to the use of samples of the derivative which were too small.

Van Slyke Amino Nitrogen Determinations—Tobacco mosaic virus in sufficiently high concentrations in acetic acid solution yields a stiff gel when sodium nitrite is added. Hence, the manometric procedure of Van Slyke (8), which permits an efficient mixing of reagents, was employed in preference to the volumetric procedure for the amino nitrogen determinations. The analyses were carried out at 24° and digestion with the nitrous acid was allowed to proceed for 20 minutes. At the end of this time, the liberation of nitrogen approached an amount corresponding to 0.13 ± 0.01 per cent amino nitrogen. The slight further liberation of nitrogen which took place almost indefinitely with time of reaction was considered to be of doubtful significance.

Tyrosine and Tryptophane Determinations—The extent of acetylation of tyrosine and tryptophane in the virus was followed colorimetrically by means of Folin's phenol reagent, as applied to unhydrolyzed proteins by Herriott (6). For the pH 8 method, samples of 0.5 to 1.0 mg. of virus in 1 cc. of solution were treated successively with 2 cc. of 50 per cent urea, 0.2 cc. of Folin's phenol reagent, and 1 cc. of alkaline phosphate. The alkaline phosphate was made up of 75 parts of 0.4 M Na_2HPO_4 , 14 parts of 10 per cent NaOH, and 11 parts of water. The phenol reagent was diluted before use, so that 0.2 cc., together with the other reagents, gave a final pH of 8.2. The urea was essential for the prevention of turbidity. Color intensities were read in the photoelectric colorimeter at some definite time after the solutions had stood for 20 minutes. It was important that all samples in a given determination be measured after exactly the same interval of time because of the fact that, as shown by Herriott, the color intensity increases almost indefinitely under these conditions. For the pH 11 method, 1 cc. samples of virus were treated with 0.1 cc. of 0.2 N NaOH for 15 minutes and then neutralized with 0.1 cc. of 0.2 N HCl before the other reagents were added. For the determination of tyrosine plus tryptophane on hydrolyzed virus, 25 mg. samples of the virus were heated for 6 hours with 1 cc. of 6 N NaOH and diluted to 25 cc. Analyses according to the procedure outlined above were then carried out on 0.74 cc. aliquots neutralized with 0.26 cc. of 1.0 N H_2SO_4 . The unknowns were compared with standard solutions containing 0.035 and 0.070 mg. of tyrosine.

Acetic Acid Determinations—The hydrolysis method of Sandor and Tabone (9) was employed instead of that of Herriott (6) because of the shorter time required in the former. 100 mg. of virus protein were heated in 2 cc. of 0.5 N NaOH for 8 hours at 100°. The hydrolysate was transferred to a 50 cc. modified Claisen distilling flask, 2 cc. of 1.0 N citric acid and 2 drops of capryl alcohol were added, and the volatile acid which was liberated was distilled *in vacuo* at a bath temperature of about 40°. The distillate was collected in a receiver which was immersed in an ice bath and was titrated with 0.01 N NaOH. Phenolphthalein was used as the indicator. 4 cc. of water and an additional drop of capryl alcohol were added to the residue in the distilling flask and the distillation and titration were repeated. This operation

was carried out six times in all. Under these conditions, controls of 0.6 mg. of acetic acid were practically completely recovered. The blank titration amounted to 0.02 cc. of 0.01 N NaOH. In the case of the hydrolysates of either normal or acetylated virus, traces of volatile acid were liberated almost indefinitely when the distillations were repeated. However, the titration value approached a more or less constant value of 0.10 cc. of 0.01 N NaOH after the sixth distillation. Errors due to the blanks for these titrations were automatically eliminated when the total titration value for the normal virus was subtracted from that for the acetylated virus.

Activity Measurements—Determinations of specific virus activity were carried out according to the directions outlined by Loring (7). Both *Nicotiana glutinosa* and *Phaseolus vulgaris* plants were used for the tests. The activity data for each virus preparation were based on results obtained on at least forty half leaves. During the winter months, when lesion counts on *Phaseolus vulgaris* plants kept in the greenhouse tended to be low, the plants were placed in a warm room at 35° for a period of 24 hours after inoculation and then returned to the greenhouse for 2 or 3 days before the lesions were counted. By this procedure, which has been resorted to by Spencer and Price (26), the average lesion count per half leaf was considerably increased.

Ultracentrifuge and Electrophoresis Measurements—Determinations of the sedimentation constant and tests for homogeneity with regard to particle size were made in the air-driven type of ultracentrifuge. The measurements were carried out on 0.3 per cent protein in 0.1 M phosphate buffer at pH 7.1.

Electrophoresis experiments were carried out in a Tiselius apparatus equipped with the Longsworth schlieren optical system (13). Preliminary measurements were made with solutions of 0.2 per cent virus protein in 0.1 ionic acetate buffer at pH 5.1. Considerable difficulty in obtaining satisfactory boundaries was encountered at first. It was found that the mechanical movement of the boundary, whether due to the action of the current or the compensating device, caused an apparent streaming of virus particles and distortion of the boundary. This phenomenon went hand in hand with the tendency for gel formation in the solutions. The acetate ion in particular seemed to cause this structure forma-

tion, since, when four-fifths of the acetate concentration was substituted by NaCl, the effect was diminished. The difficulty was largely eliminated when a change was made to 0.1 ionic K_2HPO_4 - KH_2PO_4 -KCl buffer at pH 7.3 in which 80 per cent of the ionic strength was provided by the KCl, and when the boundary was allowed to move at a relatively slow rate of 1 cm. per hour.

SUMMARY

Acetyl and phenylureido derivatives of tobacco mosaic virus have been prepared. Ultracentrifuge and electrophoretic measurements carried out on the derivatives indicated a homogeneity comparable to that of the unaltered virus.

Determinations of specific virus activity revealed that around 70 per cent of the amino groups of the virus could be covered with either acetyl or phenylureido groups without significant inactivation of the virus. Further coverage was accompanied by loss of activity. 20 to 40 per cent and possibly more of the phenol plus indole groups also could be covered without inactivation. Primarily the phenol, and not the indole, groups were affected in the above reactions.

When samples of the derivatives were inoculated into Turkish tobacco plants and allowed to propagate, normal virus was formed. It was concluded that 70 per cent of the amino groups and 20 to 40 per cent of the phenol groups in the tobacco mosaic virus molecule are not important to the basic reactions of virus reproduction.

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HEAT CURE OF ASTER YELLOWS IN PERIWINKLES

By L. O. KUNKEL

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

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In a former paper the writer reported cure of peach yellows by heat treatments (Kunkel, 1936) and showed that cured trees were virus-free. This led to the conclusion that cures resulted from thermal inactivation of yellows virus (*Chlorogenus persicae* H. var. *vulgaris* H.) present in the peach cells. Little peach, red suture, and rosette diseases of peach also were cured.

Early attempts to cure aster yellows in the China aster and in *Nicotiana rustica* L. failed, although heat treatments lasting four and eight days, respectively, brought some improvement in the health of yellows plants of both species (Kunkel, 1937). Experiments with the leafhopper that spreads aster yellows showed that infective individuals lost ability to transmit when exposed to a temperature of from 31 to 32°C. for twelve days or longer (Kunkel, 1937, 1938). That leafhoppers which were given this treatment were not incapacitated as potential vectors was proved by showing that they regained ability to transmit when allowed to feed on yellows plants. This led to the conclusion that loss of ability to transmit following exposure to heat resulted from thermal inactivation of the virus in the bodies of the leafhoppers. It also was shown that virus-bearing leafhoppers lost ability to transmit when treated at about 32°C. for periods of from one to eleven days, but that in these instances the loss was temporary. The leafhoppers so treated regained ability to transmit without having to feed on yellows plants if they were held at about 25°C. However, they did not regain ability to transmit immediately, and the time needed to reacquire this ability varied with the length of the treatment to which any given colony had been subjected. In interpreting the results of these experiments, it was assumed that treatments lasting twelve days or longer caused inactivation of all virus present in the insects, while treatments lasting from one to eleven days caused inactivation of only a part of the virus. A treatment lasting one day would be expected to inactivate a relatively small amount of virus and a treatment lasting eleven days would be expected to inactivate almost but not quite all of it. Since the time required for heat-treated insects to regain ability to transmit increased with the length of the treatment to which they had been exposed and since, after regaining ability to transmit, heat-treated insects were as effective in spreading yellows as were

untreated insects, it was concluded that the virus must multiply in the bodies of the leafhoppers. Recently, additional evidence in support of this conclusion was obtained by Black (1941), who showed, by an insect-inoculation technique, that the infectivity of juice derived from crushed leafhoppers increased during the incubation period in this host. The heat-treatment experiments conducted with infective aster leafhoppers and with yellows peach trees suggested that aster yellows might be cured by high-temperature treatments. However, when aster plants were exposed to moderately high temperatures for even a few days, most of them were severely injured by *Fusarium* wilt or other fungus diseases. A small number of plants that survived treatment died before they had time to show well-marked indications of recovery. Therefore, a search was made for a plant that might prove to be more suitable for use in heat-treatment experiments.

After a few preliminary tests, the periwinkle, *Vinca rosea* L., was selected. It was found to be a vigorous grower, very susceptible to aster yellows, resistant to fungus diseases, and capable of enduring prolonged heat treatments. Preliminary experiments showed that yellows periwinkle plants could be cured. In the work that followed, studies on inactivation of yellows virus (*Chlorogenus callishephi* H. var. *vulgaris* H.) in periwinkles was guided and supplemented by results from heat-treatment experiments with viruliferous aster leafhoppers. Some of the results obtained are reported in this paper.

Methods.—The periwinkle plants that were used were derived from a single plant by vegetative propagation. They were grown from cuttings in flats filled with equal parts of sand and peat and were transferred when a few inches high to rich earth in four-inch pots. After reaching a height of about ten inches they were transplanted to six-inch pots, and when they had outgrown these they were put in eight-inch pots. When the plants became pot-bound in eight-inch pots they were re-potted without transfer to larger vessels.

Infection of plants was obtained by two- to three-day exposures in cages containing several hundred infective aster leafhoppers. At the end of such a period, plants were transferred to a laboratory and then somewhat later to a greenhouse free of insects. They were held in the laboratory in order to insure against the possibility that one or more leafhoppers might be carried to the greenhouses if plants were transferred directly from the cages. While in the laboratory, the plants were placed near a window and were shaken from time to time. Insects that went to the window were caught and destroyed. The greenhouses in which exposed plants were kept were fumigated at frequent intervals in order to kill nymphs that hatched from eggs. When symptoms appeared, which was usually about three weeks after the plants were exposed, the date was recorded on a label placed in each pot. Heat treatments were not given until symptoms of yellows had appeared in all branches of the plants to be treated.

In general, the same methods employed in the cure of peach trees were used

in the periwinkle experiments. Plants were treated either by placing them in a previously described hot room or by immersing them in an electrically heated water-bath. The temperature of the bath was controlled to within a small fraction of a degree. The water was stirred continuously by means of a motor-driven propeller. As the bath was only eighteen inches deep, it was necessary to tie down the tops of tall plants to keep their tips under water. Preliminary experiments showed that submersion in water at room temperature for longer than one day caused some injury. Therefore, all water-bath treatments were limited to 24 hours or less.

Hot-room treatments.—The work with peach yellows showed that it was more difficult to inactivate virus in thick stems and branches than in slender twigs and much more difficult to inactivate it in the roots than in the tops of potted trees. Since a period of twelve days at about 32°C. was required to inactivate virus present in the bodies of small insects, it was surmised that at this temperature virus would be destroyed very slowly in the stems and roots of potted plants. As experiments requiring long periods of time yield information slowly, it was hoped that periwinkle plants would endure temperatures considerably higher than 32°C. Preliminary tests demonstrated that this hope was justified. Both healthy and diseased periwinkles six to eight months old endured temperatures up to 42°C. for two weeks without serious injury. After establishing this fact, a test was made to determine the effect of treatments at 42°C. on virus in the aster leafhopper. This was done in order to gain some idea of the effectiveness of high temperatures in ridding the leafhoppers of virus.

Thirty viruliferous leafhoppers were divided into two groups of fifteen. Each colony was confined on a healthy aster plant for one day and each transmitted virus. One colony which was used as a check was kept continuously in a laboratory where the temperature was about 25°C. The other was placed in a hot room at 42°C. for one day and then transferred to the same laboratory in which the check colony was held. Each colony was transferred to a series of healthy aster plants at daily intervals for 33 days. One leafhopper died while in the hot room, but the numbers of insects surviving in the two colonies were approximately the same throughout the period. At the end of the test there were four insects in the check colony and three in the treated colony. All of the 33 plants on which the check colony fed took yellows, but the 33 plants on which the treated insects fed remained healthy. The experiment demonstrated that treatment at 42°C. for one day rendered viruliferous leafhoppers permanently noninfective and presumably freed them of virus. Thus, for leafhopper-borne virus a 10°C. increase in the temperature of exposure reduced, to less than one-tenth, the time needed for inactivation at 32°. This indicated that treatments at 42°C. for one or two weeks might cure aster yellows in fairly large periwinkle plants.

The next experiment was made for the purpose of testing this hypothesis.

Two plants were given treatments for each of the following periods: four, five, six, seven, and fourteen days. All plants recovered and produced normal-appearing foliage after treatment. However, symptoms reappeared after about three months in shoots produced near the ground level in all plants except those that had been treated for fourteen days. The virus that persisted in the roots of plants treated for from four to seven days must have moved up very slowly, for no symptoms appeared in any of these plants during the first 2½ months. The plants that were treated for fourteen days were kept under observation for one year. During this time they grew vigorously, blossomed and fruited. At no time did they show symptoms of yellows in the growth produced after treatment. At the end of this period, eighteen cuttings were made from each of the cured periwinkles. All of the cuttings rooted and produced plants which were kept under observation for seven months. They could not be distinguished from plants grown from cuttings of periwinkles that had not been diseased.

The possibility that a highly attenuated strain of yellows virus might be present in the cured plants without causing easily detectable symptoms of disease was considered. In case such a virus should be present, it was thought that it might cause onset symptoms that would be more easily detected than the chronic symptoms when transmitted to healthy periwinkle or aster plants. Accordingly, three scions from each of the cured plants were grafted onto six healthy young periwinkles. The scions grew vigorously but no symptoms of disease appeared in any of the plants during the three months that they were kept under observation.

An attempt was also made to obtain transmission from cured periwinkles by means of aster leafhoppers. Colonies consisting of 35 virus-free leafhoppers each were allowed to feed for three days on each of the cured plants, on each of two yellows periwinkles, on a healthy aster plant, and on a yellows aster plant. At the end of the three-day period, each colony was transferred to a healthy aster plant and then subsequently at intervals of three to eleven days to a series of healthy aster plants. The results obtained are shown in table 1. The leafhoppers that were allowed to feed on the yellows periwinkles and on the yellows aster plant transmitted typical yellows. Those that fed on cured periwinkle and on the healthy aster plant transmitted no disease. There was no difference in the appearance of aster plants exposed to leafhoppers that had fed on cured plants and those exposed to leafhoppers that had fed on the healthy plant. The test proved that nonviruliferous leafhoppers were unable to obtain any disease-producing virus from cured periwinkles under conditions that permitted transmission from yellows aster and periwinkle plants.

This experiment brought further evidence that cured plants were virus-free. However, an immunization experiment was undertaken, because it was believed that this offered the most dependable means known for detecting whether or

not an attenuated strain of virus might be present. The plan of the experiment was to determine whether plants produced from cuttings of the cured plants would be protected against typical yellows. Eighteen periwinkles grown from cuttings from the two cured periwinkles and five healthy plants were exposed to viruliferous leafhoppers for three days. Twenty-seven days after the exposures were completed, all of the 23 plants had yellows. The plants grown from cuttings of cured plants came down as promptly and were as severely affected as the five check plants. If an attenuated strain of yellows virus had been present in any of these plants, they should have been protected. Thus, still further evidence against the hypothesis that cured plants might carry an attenuated strain of virus was secured. Since no symptoms of

TABLE 1
Yellows not Transmitted from Cured Periwinkle Plants

Successive periods on healthy aster plants	Leafhopper colony from					
	Cured periwinkle	Cured periwinkle	Yellows periwinkle	Yellows periwinkle	Healthy aster	Yellows aster
11 days	— ^c	—	—	—	—	—
5 days	—	—	+ ^d	—	—	(2) ^b
3 days	—	—	+	—	—	+
5 days	—	—	+	+	—	+
5 days	—	—	+	+	—	+
3 days	—	—	+	(1) ^a	—	+

^a (1) = Insects all died.

^b (2) = Plant died.

^c — = No transmission.

^d + = Yellows transmitted.

disease developed when scions from the two cured plants were grafted to healthy plants or when leafhoppers that had been allowed to feed on these plants were subsequently confined on healthy plants, and since plants grown from cuttings of the cured plants were not protected against yellows, it was concluded that the cured plants in question were virus-free and that heat treatment had cured them by destroying all virus present in their tissues.

Further hot-room experiments were carried out in order to obtain information as to what temperatures and what periods of treatment would be most effective and practicable. In one experiment the temperature of treatment was adjusted to about 38°C. The object was to determine whether a treatment at this temperature lasting two weeks would cure the roots as well as the tops of potted plants and whether a treatment lasting from five to seven days would cure the tops. Eighteen yellows periwinkle plants were placed in the hot room. Three similar plants were held in a greenhouse to serve as

checks. Three plants were transferred from the hot room after five days, three after six days, and three after seven days. The other nine plants were kept in the hot room for two weeks. All of the plants recovered and produced healthy-appearing foliage. Cuttings taken from the top of each of the treated plants were rooted. They all produced healthy plants. Three months after the treatment was ended, the nine plants held in the hot room for one week or less showed symptoms of yellows in shoots produced near the ground level. The other nine plants remained healthy during a period of ten months that they were kept under observation. It was concluded that these plants had been cured.

Since the tops of all treated plants used in the preceding experiment were cured, it seemed desirable to test the effects on tops of lower temperatures and shorter exposures. The next temperature chosen for a treatment was 36°C. Twenty-eight diseased plants growing in six-inch pots were given treatment. Two similar plants were left in a greenhouse to serve as checks. All of the 28 plants to be treated were placed in the hot room at the same time. Two were taken from the room and placed in a greenhouse daily beginning one day after treatment was started. After all plants had been transferred to the greenhouse, they were held under observation for 4½ months. The plants treated for one, two, three, four, and five days never produced healthy-appearing new foliage. However, the first leaves produced after treatment by the plants treated four and five days were distinctly less chlorotic than comparable leaves of check plants. The tops of the plants that were treated for six and seven days were partly cured; small branches produced normal green leaves, but yellows shoots grew from large branches. The plants treated for eight days at first produced only normal foliage. After about six weeks, several yellows shoots grew out from the main stem of one of these plants; the other plant remained healthy in appearance for about three months, when a few yellows shoots developed on the main stem three inches above the ground level. The plants treated for nine and ten days produced normal foliage for about 2½ months; then a few yellows shoots appeared on the lower portions of their main stems. The plants treated for 11, 12, 13, and 14 days likewise produced normal-appearing foliage for some time following treatment. After about three months, a few yellows shoots developed on the lower portions of their main stems. Thus, none of the treated plants in this experiment was freed of virus throughout. The symptoms that appeared in those treated for 11, 12, 13, and 14 days developed only after virus had had time to move upward from the underground parts. Apparently the virus present in the tops of these plants was inactivated. All plants treated for ten days or less retained virus in some or all of their stems and branches. The experiment showed that at 36°C. treatment of at least six days was required to cure small branches.

A comparison of the effects of heat treatments at 36°C. on virus in diseased

plants and in adult leafhoppers seemed desirable at this point, so colonies of the aster leafhopper were heat-treated by confining them in cages in the hot room for from one to eight days. The results obtained are shown in table 2.

The treatments were carried out in two separate experiments. In the first, colonies of fifteen insects each were treated for periods varying from one to six days. An untreated colony of the same size served as a check. When the information yielded by this test became available, the second experiment was planned. In this, colonies consisting of five insects each were used. They were treated for periods of seven and eight days. An untreated colony of the same size served as a check. The table shows that the different colonies were satisfactorily maintained insofar as numbers of insects were concerned. The lengths of the incubation periods shown by plants exposed to the check colonies were fairly uniform over the entire period. The plants exposed to these colonies on the first day of the test required incubation periods of eleven days each. Those exposed on the thirty-first day required an incubation period of twelve days each. This suggested that the colonies were transmitting an abundance of virus on the thirty-first day, in spite of the fact that the colony originally composed of fifteen insects had been reduced to two, and the colony originally composed of five insects had been reduced to four. Apparently there was little or no falling off in the concentration of virus in the check insects that lived to the end of the 31-day period. The average incubation period for the plants exposed to the check colonies was 10.6 days. It will be seen that eight days of treatment were required to render the insects permanently noninfective and that, when periods shorter than eight days were employed, length of treatment determined the time required for the insects to regain ability to transmit. A somewhat longer treatment was needed to render the insects permanently noninfective than had been expected, but otherwise the results obtained were anticipated. As was the case with insects treated at 32°C., delay in regaining ability to transmit varied directly but not proportionally with length of treatment. Treatments lasting one and two days caused inability to transmit for one and three days, respectively, and treatments lasting six and seven days caused inability to transmit for thirteen and twenty-three days, respectively.

The foregoing experiments showed that at 36°C. less time was needed to inactivate aster yellows virus in leafhoppers than in the tops of large plants. Eight days were required to inactivate all virus in the leafhoppers and eleven days to inactivate all virus in the aboveground parts of plants. This was anticipated, for the bodies of the insects are small and no doubt reach the temperature of the surrounding air more quickly than do large stems. Moreover, the transpiration stream probably cooled the plants until the water in the soil reached the temperature of the hot room.

Hot-water treatments.—The chief disadvantage of curing yellows in the hot

room was the length of the period of treatment. It was hoped that the disease might be successfully treated in a much shorter time by submerging plants in hot water. However, tests of the hot-water method proved that this hope was not well founded. Preliminary experiments showed that plants would not survive water-bath temperatures higher than about 45°C. for even a few hours. They also showed that short treatments at temperatures under 40°C. were ineffective. In a typical experiment, twelve yellows plants were treated by submersion in water at 45°C. Two plants were treated for each of the following periods: 1, 1½, 2, 2½, 3, and 3½ hours. The plants treated for 1½ hours were only slightly injured but were not cured. Those treated for two hours suffered considerable injury and were not cured. The plants treated for 2½ hours were severely injured, and one was cured but the other was not cured. The plants held in the bath for three hours survived treatment and were cured, but they were killed back almost to the ground level. New tops grew from shoots produced on the lower portions of the stems. The plants treated for 3½ hours were killed.

The most favorable hot-water temperature tested for cure of yellows was 40°C. Fairly large, slow-growing plants endured this temperature for 24 hours, and a good percentage of them were cured. However, some plants retained virus in their roots. In one experiment, six plants were held in the water-bath at 40°C. for 24 hours. Four of the plants were cured, but two retained virus in their roots. This treatment was very effective in the cure of aboveground parts but could not be relied upon for cure of roots. Although the hot-water treatments were successful in many instances, they were abandoned because better and more dependable results were obtained with long hot-room treatments.

Cured periwinkle plants usually began to show signs of recovery within four or five days after treatment. The first indication of cure usually appeared in small leaves near the tips of stems. The edges and ends of these leaves turned green in color. Products resulting from inactivation of virus apparently had no deleterious effects on heat-treated plants. Aster yellows in the periwinkle caused abnormal production of secondary shoots, chlorosis of stems and leaves, greening and malformation of flowers, stunting, and sterility, but all of the new growth produced after a successful heat treatment was free from these effects. As might have been expected, symptoms of yellows persisted in mature tissues. The disease subsided immediately after treatment and there were no continuing effects of any kind on the descendants of the sick cells. Plants grown from cuttings of cured plants were normal in every respect.

Susceptibility of cured plants.—It was thought that cured periwinkles might be resistant or even immune to yellows. However, as was reported for the plants produced from the two that were cured in the first hot-room experiment

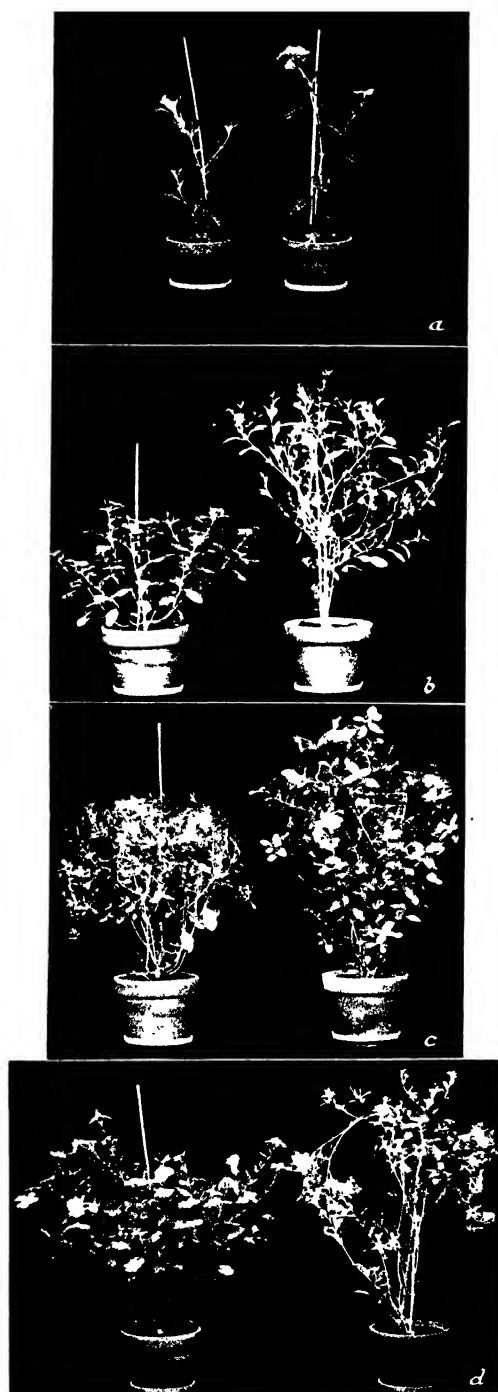


FIG. 1

described above, this did not prove to be the case. When exposed to infective leafhoppers, cured plants or plants produced from cuttings of cured plants were found to be as susceptible as those that had never been diseased. Periwinkles could be infected and cured as many times as desired. Two that were used in an early experiment were infected and cured alternately six different times. This sequence no doubt could have been repeated indefinitely, if there had been any point in continuing the cycle.

Infection, cure, reinfection, and re-cure were demonstrated in some plants that were photographed from time to time during a period of a little more than a year. Four of the photographs are presented in figure 1, which shows the plants as they appeared on four selected dates. Plant 1 and plant 2 stand at the left and at the right, respectively, in each of the photographs. Brief case histories of the plants account for the symptoms shown in the pictures. From the time the plants were grown from cuttings up to April 11, 1940, both were healthy. From the 11th to the 14th of April, plant 1 was exposed to infective aster leafhoppers. It came down promptly with yellows and is shown in the picture marked "a" as it appeared 27 days after exposure. Plant 2 was healthy at this time. On the day following that on which picture "a" was taken, plant 1 was treated by immersing it in a water-bath at 45°C. for three hours, while plant 2 was exposed to infective leafhoppers. The plants were held in a greenhouse after the treatment. Plant 1 was killed back to within four inches of the ground level by the hot-water treatment, but it was cured of yellows. Plant 2 came down with yellows about three weeks after exposure. The picture marked "b" shows the plants as they appeared four months after these treatments. It will be seen that plant 1 had normal foliage and had started to blossom, while plant 2 had yellows foliage and had produced no normal flowers. Shortly after the picture marked "b" was taken, plant 1 was exposed to infective leafhoppers for seven days, while plant 2 was given a hot-room treatment at 38°C. for twenty days. About three weeks after exposure, plant 1 came down with yellows, and a few days after the hot-room treatment plant 2 began to recover. The picture marked "c" shows the plants as they appeared about three months after the respective treatments were begun. It will be seen that plant 1 was badly diseased, while plant 2 was well on the road to recovery. It had produced many normal leaves and flowers. The size and position of some of the branches were all that remained to suggest that this plant had once had yellows. A short time after the picture marked "c" was taken, plant 1 was given a hot-room treatment at 42° for two weeks

FIG. 1. Two plants that were infected and cured of aster yellows alternately during a period of a little more than one year. The plant shown on the left in each picture was infected twice and cured twice, while that shown on the right was infected twice and cured once. (Photographs by J. A. Carlile.)

and plant 2 was exposed to infective leafhoppers for five days. By these treatments, plant 1 was cured a second time and plant 2 was given yellows a second time. The picture marked "d" shows the plants as they appeared approximately four months after the treatments. Once again plant 1 had



FIG. 2. Two *Nicotiana rustica* plants that were equally affected by yellows before the plant on the left was cured by a heat treatment given two months before the picture was taken. (Photograph by J. A. Carlile.)

normal foliage and flowers, and once again plant 2 was severely diseased. The pictures demonstrate the fact that the plants were infected and cured at will.

Since cured plants were usually exposed to infective leafhoppers several months after they had been cured and after they had made considerable new growth, reinfection did not prove that the plants failed to gain temporary immunity by having had yellows. In order to determine whether tissues that

had been diseased and cured were immediately susceptible to reinfection, the following experiment was made. Four yellows plants were cured by treatments at 42°C. for two weeks. On removal from the hot room, two of the plants were at once exposed to infective leafhoppers; the other two were not exposed and served as checks. The check plants remained healthy but the exposed plants came down with yellows. This and other similar experiments proved that cured plants were as susceptible to infection as plants that had not had yellows.

Cure of aster yellows in other plants.—After some experience had been gained in the cure of periwinkles, a second attempt was made to cure yellows in *Nicotiana rustica*. Diseased plants that were somewhat older than those treated previously were held in the hot room at 40°C. for two weeks. These plants were cured in the tops, but virus persisted in the roots. In another experiment, plants were held at 40°C. for three weeks, and these were cured in the roots as well as in the tops. Two plants from this experiment are shown in figure 2. They were equally affected by yellows before the plant on the left was treated. The picture was made two months after the treatment ended. It shows that the treated plant had recovered and was flowering and fruiting normally, while the untreated plant was badly diseased.

Attempts were also made to cure aster yellows in lettuce. Yellows plants of several different varieties were heat-treated at 38° and 40°C. Most of the plants died of fungus infections during the first week of treatment. The plants that remained were removed to a greenhouse. The tops of the plants were cured, but virus persisted in the roots. Some of these plants flowered and fruited. However, it is believed that fungus infections must be controlled before it will be possible to treat yellows lettuce plants successfully.

Discussion.—In combating virus diseases, phytopathologists have emphasized prevention rather than cure. Nevertheless, some cures have been reported. Baur (1906, 1906a) found that he was able to cure mosaic-diseased *Abutilon* plants by shading or by cutting off chlorotic leaves or parts of leaves over a period of time. Wilbrink reported in 1923 that she could cure sugarcane seed pieces of serah disease by immersion in water held at about 52°C. for about one hour. Chlorotic streak of sugarcane was cured by a similar treatment (Martin, 1932). As was mentioned in the introduction to this paper, Kunkel (1936) used heat to cure peach trees of yellows, little peach, red suture, and rosette. Three years later, Hutchins and La Rue (1939) stated that promising results were secured by heat-treating peach trees affected by phony disease, and quite recently Hildebrand (1941) has stated that he obtained encouraging results in studies on heat inactivation of the virus of the yellow-red disease of peach.

Thus, *Abutilon* mosaic has been cured by shading and by amputation of diseased leaves. Two sugarcane diseases presumed to be caused by viruses

and four peach virus diseases have been cured by appropriate heat treatments. In addition, two other peach virus diseases have been reported to have responded favorably to heat. To this list may now be added aster yellows disease in the periwinkle, *Vinca rosea*, and in *Nicotiana rustica*.

Aster yellows affects many different species of plants, including several that are of economic importance (Kunkel, 1926, 1931). The disease can probably be cured in all species capable of enduring moderately high temperatures for prolonged periods of time. The higher the temperature that can be endured by any plant, the more practicable will be the heat-treatment method for cure of that plant. All species capable of withstanding temperatures up to 38°C. for two weeks probably can be cured. Cure of field-grown plants by heat does not seem feasible at present, but cure of certain commercial greenhouse plants and plants of special value for breeding purposes should be practicable.

In the experiments reported, mention was made of the fact that a considerable number of yellows periwinkles that were not cured were favorably affected by the heat treatments. It is presumed that the favorable effects resulted from inactivation of a part of the virus present in these plants. Two patterns of recovery short of cure were observed. In the one, all virus present in the aboveground parts, but not all of that present in the roots, was inactivated. In the other, some virus remained in both tops and roots. The latter type of recovery was slight and transitory, but the former type was highly beneficial. Apparently yellows disease in roots did not interfere seriously with absorption of water and nutrients from the soil, for plants with cured tops but diseased roots made good growth and blossomed profusely. Moreover, yellows virus moved from roots to tops at such a slow rate that the favorable effects of curing the tops lasted for many months.

The destructive action of high temperatures on yellows virus *in vivo* may be either direct or indirect. The gradual disappearance of virus in, and final absence of virus from, both plants and insects held at high temperatures suggested thermal inactivation. Whatever the action may have been that destroyed virus in insects and plants, it ceased and was replaced by virus increase when either of these hosts was brought to temperatures below the inactivation point, provided of course that the inactivating reaction had not gone to completion. When it had gone to completion, insects lost permanently their ability to transmit virus and plants were cured. However, this did not deprive insects of the ability to reacquire infectivity or protect plants against reinfection. The reaction of aster yellows virus to heat *in vitro* has not been studied because of inability mechanically to inoculate plants. The fact that it was possible to cure plants and free insects of virus by moderately high temperature treatments suggests that the thermal inactivation point is low.

We have in aster yellows virus an entity that cannot exist for long at temperatures that frequently are reached during summer months. It is able to spread only in summer and able to exist for protracted periods only at fairly low temperatures. The temperature limitation is probably the factor that prevents it from flourishing in our southern states. However, its rapid spread under appropriate weather conditions shows that it is an efficient parasite.

Nonviruliferous leafhoppers become infected by feeding on yellows plants, and healthy plants become diseased by serving as hosts for viruliferous leafhoppers. There is good evidence that the virus multiplies in the leafhoppers as well as in the plants, and that in nature it passes alternately from the one to the other. From the life history standpoint, aster yellows virus must be classified among those obligate heteroecious parasites of which the plant rusts furnish so many examples, but, whereas plant rusts alternate between plant species, the aster yellows virus alternates between plant and insect species.

All attempts to obtain serological reactions with aster yellows virus have failed (Chester, 1937). An explanation for this rather puzzling situation may be found in the instability of the virus at blood temperature.

Although aster yellows virus and tobacco mosaic virus represent extremes in respect to heat stability, they cause diseases that are remarkably similar in some ways. In plants they both produce conspicuous clearing of veins, stunting, and chlorosis. They both go to a large number of different species. However, the aster yellows virus produces a general chlorosis, whereas the tobacco mosaic virus causes mottling. Plants affected by yellows become more and more depauperate with the passing of time, but those affected by mosaic recover to a certain degree after onset.

Grainger (1939, 1940) has shown that the temperature of optimum activity of tobacco mosaic virus in tobacco is not wholly coincident with that of the host. This suggested that virus activity was more or less independent of host activity. Cure of aster yellows by heat inactivation of yellows virus in periwinkle plants and inactivation of virus in aster leafhoppers shows that, in respect to heat resistance, yellows virus reacts independently of its hosts.

SUMMARY

Yellows periwinkle plants were cured by treatments in a hot room at 38° to 42°C. for two weeks.

Periwinkles also were cured by immersion in a water-bath held at 40° to 45°C. for a few hours.

Cured plants were shown to be free of virus.

Longer treatments were required to inactivate virus in the stems of plants than in the bodies of aster leafhoppers.

It was more difficult to inactivate virus in roots than in tops of potted plants.

Periwinkles could be infected and cured as often as desired. There was no

evidence that cured plants were more resistant to yellows than plants that had never had yellows.

Yellows *Nicotiana rustica* plants were cured by treatments at 40°C. for three weeks.

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A DISTINCTIVE STRAIN OF TOBACCO-MOSAIC VIRUS FROM PLANTAGO

By FRANCIS O. HOLMES

(From the Department of Animal and Plant Pathology of The Rockefeller Institute,
Princeton, New Jersey)

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A mosaic disease was observed, in the autumn of 1940, in plants of rib grass (*Plantago lanceolata* L.) and of the broad-leaved plantain (*P. major* L.), growing as weeds in several widely separated locations near Princeton, New Jersey. Affected plants, interspersed among much larger numbers of healthy-appearing plants of the same species, were distinguished by slight twisting of petioles, chlorotic streaks along veins, and systemic chlorotic mottling (Fig. 1, A).

Inoculation of Turkish tobacco (*Nicotiana tabacum* L.) with undiluted juices expressed from affected plants of each of the *Plantago* species initiated a disease characterized by numerous primary lesions (Fig. 1, B) resembling those of tobacco-ring-spot disease. Usually these primary lesions appeared as thin, white rings of necrotic tissues surrounding green or only slightly chlorotic central areas and distributed in a random manner over the inoculated portion of the leaf lamina. Sometimes, however, they appeared as coarse, brown rings of necrotic tissues with green centers or as solid, brown necrotic spots. The primary lesions first became distinguishable on the third or fourth day after inoculation. A day or two later, comparable secondary lesions appeared in several successive uninoculated leaves at the top of each plant (Fig. 1, C). These secondary lesions consisted of rings and lines of necrotic tissues along veins; they were usually white and delicate in character but occasionally brown and coarse. Both primary and secondary necrotic lesions were sometimes zonate. Necrosis did not occur in newly formed leaves after the onset of systemic attack unless the infected plants were forced into unusually rapid growth, as by being cut back severely. Instead, the next leaves that were formed showed chlorotic mottling in which the chlorotic areas were often few and large (Fig. 1, D). As the diseased plants matured, new leaves were variously affected. Some showed a blurred chlorotic mottling; others were green and nearly normal in appearance; a few showed sharply delineated mottling in which the chlorotic areas were numerous and small.

Inoculation of *Nicotiana glutinosa* L. gave rise locally to numerous brown necrotic lesions, essentially like those produced in this host by tobacco-mosaic virus (*Marmor tabaci* H.). The lesions were slightly smaller than those char-

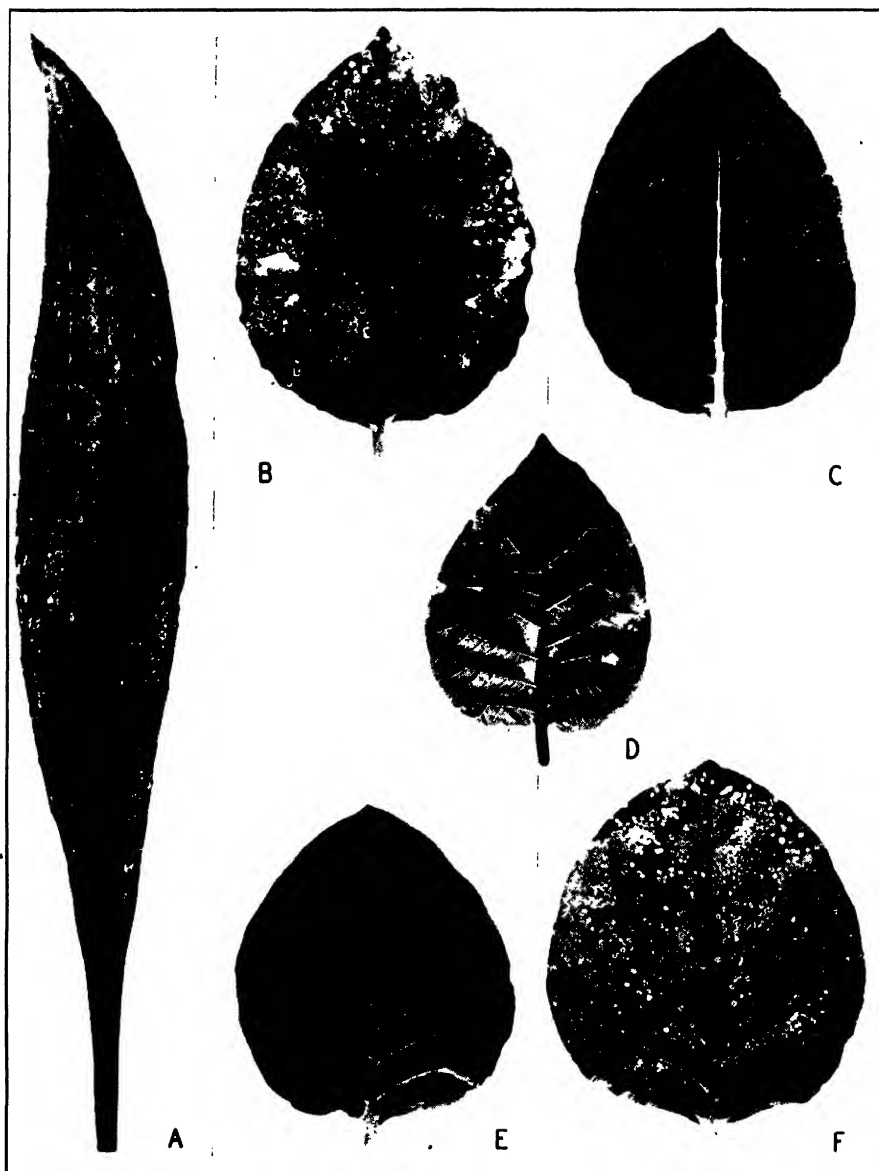


FIG. 1. Effects induced by experimental inoculation with the rib-grass strain of tobacco-mosaic virus. A. Chlorotic mottling in rib-grass, *Plantago lanceolata* L. B. White necrotic-ring primary lesions in Turkish tobacco, *Nicotiana tabacum* L. C. Similar secondary lesions and, D, subsequent chlorotic mottling in Turkish tobacco. E. Absence of primary lesions in tobacco leaf protected by earlier infection with the type strain of tobacco-mosaic virus, in contrast with, F, characteristic 5-day lesions induced by the rib-grass strain in a control leaf inoculated previously only with juice of healthy tobacco and hence not protected. (Photographs by J. A. Carlile.)

acteristic of the type strain of this virus and were less heavily pigmented peripherally; no systemic spread of the disease was observed.

The two original isolates of virus, from rib-grass and broad-leaved plantain, respectively, appeared to be identical in their effects on *Nicotiana tabacum* and on *N. glutinosa*. The strain from rib grass was used in all subsequent experiments, as being representative.

The preliminary observations already described had raised the question of whether more than one virus might be concerned in the original disease in rib grass, since no single virus was known to produce all of the observed effects. Further studies were conducted, in part to answer this question and in part to permit the identification of the virus or viruses involved.

Experimental Studies of the Rib-Grass Virus

Resistance to Heat. Preliminary tests indicated that the rib-grass virus was relatively resistant to heat. In order to appraise its stability more accurately, juice expressed from systemically infected Turkish tobacco plants, such as served as sources of inoculum for use in all subsequent experiments except as otherwise stated, was exposed in thin-walled glass tubes, without dilution, to a temperature of 85°C. for 0, 5, 10, 15, and 30-minute periods; 10-leaf tests on *Nicotiana glutinosa* gave lesion counts of 3104, 2068, 1947, 1437, and 1242, respectively, showing gradual but slight decrease of infectivity with increase of time at this temperature. Similar exposures at 90°C. gave counts of 3668, 1216, 639, 83, and 1, showing more rapid decrease of infectivity but not complete inactivation at this higher temperature. Additional tests showed that the virus was completely inactivated in 10 minutes at 93°C., not at 92°C.

Only 1 virus that has been shown to spread systemically in tobacco is known to resist exposure at 85°C. or at any higher temperatures for as long as 10 minutes without complete inactivation; this is tobacco-mosaic virus. The result of the heat treatments, in conjunction with the character of the lesions on *Nicotiana glutinosa*, gave preliminary evidence, therefore, that the rib-grass disease was caused by a strain of tobacco-mosaic virus, either alone or in mixture with some other virus. It seemed highly improbable that a second virus also could withstand this relatively high temperature treatment. Transfers were made to tobacco, therefore, from single lesions in *N. glutinosa* plants inoculated with juice that had been exposed at 85°C. for 10 minutes. These transfers induced a disease having all the characteristics of the malady earlier produced in tobacco by inoculation with crude juice taken directly from diseased rib-grass plants in the field. Thus, it appeared that all the effects observed in tobacco could be produced by a single-lesion isolate of a virus having approximately the thermal stability of typical tobacco-mosaic virus.

Resistance to Drying. A stock of the rib-grass virus in dried tobacco leaves

was stored in the laboratory at about 23°C. and was tested after 73 and 122 days by inoculation of *Nicotiana tabacum* and *N. glutinosa*. A high degree of infectiousness was noted on each occasion, and the disease type was found not to have been modified by storage of the virus in dried leaf tissues. In resistance to desiccation in leaf tissues, as in resistance to heat, the rib-grass virus resembled tobacco-mosaic virus.

Filterability. A sample of juice from an infected tobacco plant was centrifuged at low speed to remove the larger particles of cellular debris; 10 cc. of the cleared juice was then added to 85 cc. of water and 5 cc. of a 1-day-old broth culture of the red-pigment-forming bacillus, *Serratia marcescens* Bizio 1823 (= *Bacillus prodigiosus* Flüge 1886). Part of the mixture of diluted infectious juice and bacterial culture was filtered through a Berkefeld W porcelain candle; the filtrate proved to be free from bacteria, as determined by agar-plate tests of 2 samples, each of 1-cc. volume, but induced the formation of 80.2 lesions per leaf on 10 inoculated leaves of *Nicotiana glutinosa*. The filterability of the rib-grass virus was thus demonstrated. Control tests of the unfiltered mixture showed more than 1000 bacteria per cc. and a virus content sufficient to induce the formation of 64.4 lesions per leaf on 10 similar leaves of *N. glutinosa*.

Infectiousness at High Dilutions. The virus in freshly expressed juice was so concentrated that it remained infectious, even when diluted to 1:10⁶. The numbers of lesions produced on sets of 20 leaves of *Nicotiana glutinosa* inoculated with 9 successive 10-fold dilutions (10⁰ to 10⁻⁸) of juice expressed from a tobacco plant infected 91 days previously were: 9700, 6088, 1700, 236, 88, 14, 1, 0, 0. This ability to infect at high dilution is comparable to that of many of the more infectious strains of tobacco-mosaic virus and but little inferior to that of the type strain, though the leaves of the old plant used as source of virus had appeared green and nearly like those of healthy plants.

Inability to Infect Bean. Most strains of tobacco-mosaic virus cause the formation of brownish-red local lesions in inoculated leaves of Early Golden Cluster bean (*Phaseolus vulgaris* L.) (9), although a strain which appears incapable of producing lesions in this plant has been described by Melchers *et al.* (8). Repeated attempts to transmit the rib-grass virus to Early Golden Cluster and other varieties of bean have resulted in failure. Apparently *P. vulgaris* is immune from this virus under the conditions of the experiments. Because of this characteristic, it has been possible to show by the inoculation of bean leaves that samples of the rib-grass virus used in the tests reported here did not owe their tobacco-mosaic-like characteristics to contamination with ordinary tobacco-mosaic virus, which, if present, would have been disclosed by the development of characteristic lesions.

Effect on *Nicotiana sylvestris*. Dark-brown, necrotic, local lesions alone appeared on *Nicotiana sylvestris* Speng. and Comes plants that had been inocu-

lated with the rib-grass virus. In this respect the virus resembled the so-called aucuba types of tobacco-mosaic virus, a group of substrains and isolates collectively constituting the tomato aucuba-mosaic strain of this virus (*Marmor tabaci* var. *aucuba* H.).

Response to Presence of Gene N in Tobacco. Further diagnostic evidence was obtained by direct transfer of virus from the spontaneous disease in rib grass to a segregating progeny of tobacco plants, some bearing the dominant gene *N* from *Nicotiana glutinosa*, others its recessive allele *n*. Plants bearing the dominant gene *N* respond to infection with typical tobacco-mosaic virus by forming necrotic lesions of the type characteristic of *N. glutinosa* (5), whereas plants lacking it respond by forming extremely faint chlorotic primary lesions that are sometimes difficult to detect without staining in iodine solutions (3) and by showing subsequent chlorotic mottling. The virus from rib grass also produced 2 types of disease, in some plants the brown, necrotic, local lesions of *N. glutinosa* type, and in others primary and secondary thin, white, necrotic, ring-like lesions with green or slightly chlorotic central areas, followed by mottling, as in ordinary tobacco infected with this rib-grass virus. The results of this test tended to confirm the earlier evidence that a strain of tobacco-mosaic virus was present in the diseased plants, for no other virus is known to be so influenced by the gene *N* that 2 sharply distinguishable disease types are produced. Moreover, these results confirmed the evidence already available that but 1 virus was responsible for all the observed effects. Had a second virus been responsible for the necrotic rings in ordinary tobacco, the *N*-type tobacco plants used in this experiment should have shown not only the brown necrotic spots, characteristic of infection by tobacco-mosaic virus, but also the primary and secondary ring-like lesions.

Immunological Relationships. Tobacco plants inoculated over the whole surface of a leaf with the type strain of tobacco-mosaic virus 5 days before inoculation of the same leaf with the rib-grass virus developed no necrotic lesions, whereas tobacco plants of the same age inoculated with the rib-grass strain 5 days after preliminary inoculation with juice from healthy tobacco plants developed large numbers of the characteristic necrotic lesions (Fig. 1, E and F). Furthermore, tobacco plants, systemically infected with typical tobacco-mosaic virus, did not show necrotic primary lesions as a result of subsequent inoculation with the rib-grass virus. This protection, as a result of previous infection with a strain of tobacco-mosaic virus known not to cause necrosis, confirmed the diagnosis of tobacco-mosaic virus as wholly responsible for the necrotic-ring effects in tobacco. No such protection was conferred on tobacco plants by previous infection with other mosaic viruses, such as cucumber-mosaic virus (*M. cucumeris* H.) and alfalfa-mosaic virus (*M. medicaginis* H.).

Confirmation of the protection tests in tobacco was given by experiments

in which it was demonstrated that protection was afforded to parts of leaves of *Nicotiana sylvestris* by inoculations with typical tobacco-mosaic virus 5 days before their inoculation with the rib-grass virus. No necrotic lesions appeared in areas already invaded by the type virus, but many necrotic lesions appeared in uninoculated areas or in areas rubbed only with juice of healthy tobacco in the preliminary inoculation. Kunkel (6) has shown that this type of test is an excellent criterion for determining the possible relationship of a virus to known strains of tobacco-mosaic virus.

Serological Relationships. Characteristic flocculent precipitates were secured with the rib-grass virus in the presence of antiserum to the type strain of tobacco-mosaic virus. The samples of rib-grass-mosaic virus used in this serological test were shown to be highly infective, typical, and free from contamination with ordinary tobacco-mosaic virus by inoculation of *Nicotiana glutinosa*, *N. tabacum*, and *Phaseolus vulgaris*. Large numbers of lesions in the first of these species reflected the high titer of the sample in hand, necrotic-ring production in the second showed the nature of the strain to be unmodified, and complete lack of lesions in the third showed the absence of ordinary tobacco-mosaic virus, control inoculations with which produced abundant reddish necrotic lesions, as usual, in accompanying bean plants grown simultaneously and maintained under the same conditions in the greenhouse.

Morphology. Electron micrographs, made by Thomas F. Anderson, Fellow of the National Research Council at the laboratories of the Radio Corporation of America in Camden, N. J., showed the rib-grass-virus particles to be rod-like in form. A portion of one of these micrographs is represented in figure 2, with a scale superposed on it to facilitate estimation of particle lengths. Preparations of the rib-grass virus that had been purified by ultracentrifugation were examined by I. Fankuchen, National Research Council Fellow in Protein Chemistry at the Physics Department, Massachusetts Institute of Technology. Through the use of an X-ray diffraction technique (2), he determined the average diameter of the virus particles to be about 15.0 μ (150 Å.) in air-dried preparations. Both the rod-like shape disclosed by use of the electron microscope and the width of particles as measured by X-ray methods agreed well with the hypothesis that the rib-grass virus was a strain of tobacco-mosaic virus.

Invasiveness. The rib-grass virus differed from the type strain of tobacco-mosaic virus in its greater ability to invade plants of *Plantago lanceolata* systemically, the type strain being known to increase in the inoculated leaf (4, p. 64), but not to produce obvious disease, and not to spread to new leaves in this host; indeed, Allard (1, p. 11) reported that the most rigorous methods of inoculation were ineffective in producing any visible symptoms of disease in rib grass. Perhaps it is not surprising, therefore, that the rib-grass strain was found less able to penetrate the tissues of some solanaceous species, its spe-

cialization for *Plantago* being accompanied by a notable reduction of invasiveness in such plants as tomato and *Physalis angulata* L.

In tomato (*Lycopersicon esculentum* Mill.), the rib-grass virus produced either localized and inapparent infections, or, less often, a systemic disease characterized by few secondary lesions, constituting a spotty chlorotic mottling.



FIG. 2. Electron micrograph of the rib-grass strain of tobacco-mosaic virus reproduced at a magnification of 30,000 diameters. (Micrograph by T. F. Anderson.)

Typical tobacco-mosaic virus under the same environmental conditions regularly became systemic and caused a well-developed chlorotic mottling in this host.

In *Physalis angulata*, only primary lesions were formed by the rib-grass virus; these were mostly chlorotic in character, though a few showed necrotic peripheries; secondary lesions were absent and no defoliation occurred. Typical tobacco-mosaic virus became systemic in this host; its primary and secondary lesions were nearly all in the form of fine white necrotic rings with green

centers, a few being coarser necrotic rings or necrotic brown spots; abscission of affected leaves led eventually to complete defoliation of the plants.

Inability to Infect Cucumber. Despite the ability of the rib-grass virus to invade *Plantago* systemically, an ability not possessed by typical tobacco-mosaic virus, cucumber (*Cucumis sativus* L.) appeared to be insusceptible to infection, no disease appearing after inoculation and no evidence of virus increase being obtained on subinoculation. In this inability to infect cucumber, the rib-grass virus resembles typical tobacco-mosaic virus and differs sharply from cucurbit-mosaic virus (*Marmor astrictum* H.), which has no known hosts in common with tobacco-mosaic virus but shares its physical and chemical properties, even to the point of antigenic similarity.

Stability. The rib-grass virus is relatively stable, at least in the sense that it has not been observed to give off variants resembling ordinary tobacco-mosaic virus. Tobacco plants, infected with the rib-grass virus when young and tested more than 2 months later, showed no evidence of the development of strains of the virus capable of infecting bean plants. Ten successive transfers in tobacco failed to modify the virus enough to permit it to form lesions in the bean, to move freely in tomato, or to mottle tobacco without inducing the formation of necrotic-ring lesions. Tomato plants, infected when young, usually showed no evidence of disease to blossoming time; in a few plants there were scattered chlorotic lesions in upper leaves. Inoculated leaves of tomato plants contained much virus when tested 18 days after inoculation; the tops of the same plants, however, showed no virus in some cases and but little in others; this would not have been the case had variants resembling the type strain been formed.

Existence of Substrains. Successive isolates from diseased rib-grass plants in the field disclosed the existence of variants of the rib-grass virus. All isolates, however, produced necrotic primary lesions on transfer to Turkish tobacco plants. All were similar in being uninfecious for the Early Golden Cluster bean. One isolate was characterized by its tendency to produce necrotic rings on Turkish tobacco a little later than the others. On the whole, the similarities between the isolates were more striking than the slight differences that were detected. Because of this, the isolates thus far observed seem to constitute a closely related group and collectively to represent a strain of tobacco-mosaic virus, adapted to *Plantago* as a natural host, characterized by producing ring-like necrotic primary lesions in Turkish tobacco, and typified by the isolate described in detail in this paper.

DISCUSSION

Similarity to Recognized Strains of Tobacco-mosaic Virus. The rib-grass virus resembles to some extent a tomato-mosaic strain of tobacco-mosaic virus studied by Melchers *et al.* (8); this strain also failed to infect bean and produced only necrotic local lesions on *Nicotiana sylvestris*; it differed by producing no obvious primary lesions of any sort and no secondary necrotic-ring lesions, but

only chlorotic mottling, in "Samsun" tobacco, a variety much like the Turkish tobacco used in the present study. In at least one way, the rib-grass virus resembles also the mild dark-green mosaic virus of McKinney (7, pp. 565-566), which is possibly a strain of tobacco-mosaic virus. This virus is said not to infect tomato, and, for its part, the rib-grass strain is not easily induced to move systemically in the tomato nor to produce outward manifestations of disease in this host. The mild dark-green mosaic virus of McKinney, however, like the tomato-mosaic virus of Melchers *et al.*, does not produce necrotic-ring lesions in tobacco. Resemblance of the rib-grass virus to the tomato aucuba-mosaic strain of tobacco-mosaic virus in production of localized necrosis in *N. sylvestris* is also of but limited significance, for previously recognized, *aucuba*-type substrains cause no necrotic, but only chlorotic, effects in Turkish tobacco and readily infect the bean. The minute-lesion strain of tobacco-mosaic virus (*Marmor tabaci* var. *artum* H.), which produces even smaller lesions in *N. glutinosa* than does the rib-grass strain, causes no necrosis in Turkish tobacco and infects the bean, though its lesions tend to be small in this host as in *N. glutinosa*. On the whole, the rib-grass virus shows striking characteristics that differentiate it from previously recognized strains.

Technical Designation of the Rib-grass Strain of Tobacco-mosaic Virus. The rib-grass virus appears to deserve consideration as a distinctive strain of tobacco-mosaic virus, its identity with this virus being clearly shown by its remarkable resistance to heat and desiccation, its precipitation by an appropriate antiserum, its exclusion from tissues previously infected by typical tobacco-mosaic virus but not by other viruses, and its response to the genic constitution of tobacco. The rib-grass strain of tobacco-mosaic virus is unusual in its adaptation to rib grass, in its tendency to induce peculiar necrotic-ring effects in tobacco, and in its inability to infect the bean. In particular, the induction of characteristic necrotic-ring effects in Turkish tobacco would appear to warrant its recognition as an entity deserving varietal status, occurring in nature, consisting of closely related substrains, and typified by the isolate here described. The name *Marmor tabaci* var. *plantaginis* n. var. is hereby given to it.

There has been some adverse criticism of the application of varietal names to strains of viruses (10). This criticism appears to be based on the belief that later collections may not exactly duplicate an isolate first described as type of a variety. Would not this objection apply also to the naming of a species? Minor variations among subsequent isolates, however, if only of subsidiary character and significance, need not interfere with assignment of an isolate to an otherwise appropriate category, whether of specific, varietal, or other rank.

SUMMARY

A filterable virus, causing a mosaic disease of rib grass, *Plantago lanceolata* L., and of the broad-leaved plantain, *P. major* L. (*Plantaginaceae*), was found to produce necrotic-ring patterns when transferred experimentally to Turkish

tobacco (*Nicotiana tabacum* L.). Its failure to be inactivated by heat when exposed at 92°C. for 10 minutes, its inability to form its characteristic necrotic primary lesions in tissues of *N. tabacum* and of *N. sylvestris* Speg. and Comes, already invaded by typical tobacco-mosaic virus, its precipitation by tobacco-mosaic-virus antiserum, and its response to the genic constitution of tobacco showed that it was a strain of tobacco-mosaic virus (*Marmor tabaci* H.). This rib-grass strain of tobacco-mosaic virus, distinguished from previously known strains by its ability to form necrotic-ring lesions in tobacco, was given the varietal designation *Marmor tabaci* var. *plantaginis* n. var.

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THE REVERSIBLE INACTIVATION OF TOBACCO MOSAIC VIRUS BY CRYSTALLINE RIBONUCLEASE

By HUBERT S. LORING

*(From the Department of Animal and Plant Pathology of The Rockefeller Institute for
Medical Research, Princeton, New Jersey, and The Department of Chemistry,
Stanford University, California)*

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The inactivation of plant viruses by enzymic action has been reported by several investigators (1-5). The inactivation may be irreversible as in the case of pepsin where virus protein is hydrolyzed (3) or reversible as in the case of trypsin where the inactivation appears to be due in part to a decreased susceptibility of the test plant in the presence of the enzyme (4). Besides its protein component tobacco mosaic virus also contains a ribonucleic acid, which thus far has proved necessary for virus activity. In experiments with phosphatase Pfankuch and Kausche (5) have demonstrated inactivation of tobacco mosaic and latent mosaic viruses and, although chemical proof was lacking, suggested that the loss of activity may be due to a splitting off of a phosphate group from the virus nucleic acid. It is unlikely that phosphatase would affect the nucleic acid present in a virus particle because it has no demonstrable effect on the free nucleic acid itself (6). The crystalline ribonuclease recently isolated by Kunitz, on the contrary, splits yeast ribonucleic acid into particles which have a higher diffusion rate than free ribonucleic acid and are not precipitated by glacial acetic acid (7). It also produces a similar change in the free virus nucleic acid and depending on the type of linkage between protein and nucleic acid in the virus might be expected to split off a portion of the nucleic acid. Preliminary experiments showed that the virus was inactivated by relatively low concentrations of ribonuclease. Studies on the rate of inactivation, on the effect of different enzyme concentrations and of dilution of the inactive virus-enzyme mixtures on virus activity suggested that inactivation was brought about by a combination of virus with ribonuclease. In more concentrated solutions and in the absence of salt, the virus-enzyme complex separated in the form of long fiber-like particles, which on analysis proved to contain about 14 per cent ribonuclease. That this inactive complex could be readily dissociated to give back virus was shown by recovery of about 100 per cent of the original virus activity from an inactive virus-enzyme complex and by the solubility behavior of the complex at different hydrogen ion concentrations. This paper presents the results of these experiments.

Effect of Ribonuclease Concentration on Virus Activity.—Preliminary experiments showed that the virus activity of solutions containing from 10^{-3} to 10^{-5} gm. of purified tobacco mosaic virus per ml. was strikingly reduced by the presence of relatively small amounts of crystalline ribonuclease.¹ In order to determine the effect quantitatively solutions were made up containing 10^{-3} and 10^{-5} gm. of virus per ml. respectively and enzyme concentrations in steps of ten from 1.2×10^{-9} gm. per ml. to 1.2×10^{-4} gm. per ml. in 0.1

TABLE I

Effect of Ribonuclease Concentration on Virus Activity When Tested Immediately after Mixing

Concentration (gm. protein per ml.)		<i>Phaseolus vulgaris</i> *				<i>Nicotiana glutinosa</i> *			
Virus	Ribonuclease	No. of lesions		Concentration of control (gm. per ml.)	Per cent of original activity	No. of lesions		Concentration of control (gm. per ml.)	Per cent of original activity
		Mixture	Control			Mixture	Control		
10^{-5}	1.2×10^{-9}	4	4	10^{-7}	1.0	40	3	10^{-7}	10
	1.2×10^{-7}	39	15	10^{-7}	2.6	149	3	10^{-7}	50
	1.2×10^{-6}	135	70	10^{-8}	20	138	36	10^{-8}	40
	1.2×10^{-5}	551	394	10^{-8}	140	251	203	10^{-8}	125
10^{-3}	1.2×10^{-4}	6	6084	10^{-3}	0.1	36	312	10^{-3}	10
	1.2×10^{-3}	14	6084	10^{-3}	0.2	133	312	10^{-3}	40
	1.2×10^{-6}	317	6084	10^{-3}	5	424	312	10^{-3}	140
	1.2×10^{-7}	1489	6084	10^{-3}	25	387	312	10^{-3}	130

* With a concentration of 10^{-5} gm. virus per ml., 10 to 16 half leaves were used for each mixture and its respective control: with a concentration of 10^{-3} gm. virus per ml., 5 to 8 whole leaves were used both for the test solution and the controls.

† Calculated from the number of lesions obtained from the mixture and the control and their respective virus concentrations. Per cent original activity = $\frac{\text{No. of lesions (mixture)}}{\text{No. of lesions (control)}} \times \frac{\text{Concentration of virus (control)}}{\text{Concentration of virus (mixture)}} \times 100$.

M phosphate buffer at pH 7. The activities of these solutions were then compared immediately by the half-leaf method (8) on leaves of *Nicotiana glutinosa* L. and *Phaseolus vulgaris* L. var. Early Golden Cluster with concentrations of virus which gave approximately the same number of lesions. The activities of the solutions were then calculated as percentage activity of the original virus solutions in each case. These data which are summarized in Table I show that an enzyme concentration of 1.2×10^{-9} gm. per ml. has little or no effect on virus activity at a virus concentration of 10^{-5} gm. per

¹ The writer is indebted to Dr. M. Kunitz for the preparation of crystalline ribonuclease.

ml. In this more dilute virus solution a decrease in activity was observed with a concentration of 1.2×10^{-8} gm. ribonuclease per ml. when tested on Early Golden Cluster plants but the effect was not pronounced until a concentration of 1.2×10^{-7} or 1.2×10^{-6} gm. per ml. was reached. Some difference was observed in the behavior of the two types of test plants. As in earlier experiments with trypsin (4) the *P. vulgaris* plants were relatively more sensitive to the enzyme than *N. glutinosa* plants. With a virus concentration of 10^{-3} gm. per ml., higher enzyme concentrations were required to show inactivation, indicating in the case of the more dilute virus solutions that the inactivation was not due entirely to a decreased susceptibility of the

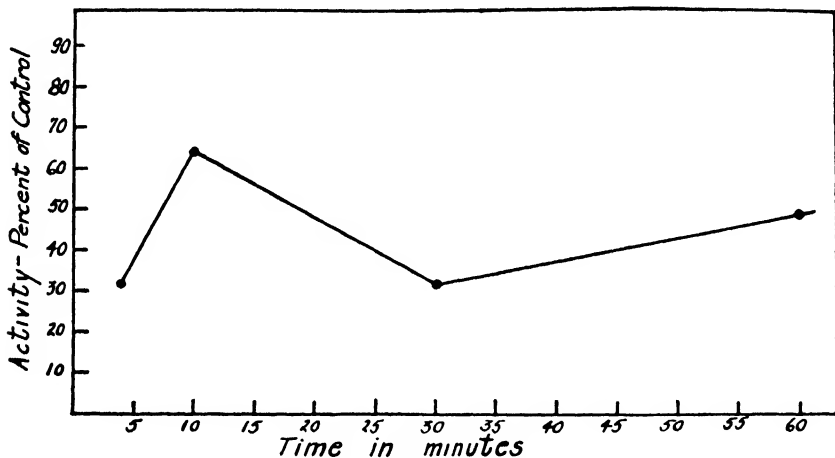


FIG. 1. Effect of time of standing on inactivation of tobacco mosaic virus by ribonuclease.

test plants in the presence of ribonuclease. In this connection it is of interest to compare the enzyme concentrations which produced inactivation with the concentrations of trypsin and those of various inert proteins which cause an inhibition of virus activity as studied by Stanley (4) and Ross and Stanley (9), respectively. The amount of ribonuclease required to cause almost 100 per cent inactivation is of the order of one-one hundredth the amount of trypsin and is about one-one hundred thousandth the amount of inactive proteins studied by Ross and Stanley (9), which produced about a 50 per cent inactivation.

Rate of Inactivation of Virus.—The question of the effect of time on the inactivation of virus was studied by comparing the activity of virus-enzyme mixtures with suitable controls immediately and after they had stood at room temperatures for varying periods of time up to 10 days. The results of a typical experiment are shown in Fig. 1. The mixture consisted of virus

at 10^{-3} gm. per ml. and enzyme at 1.2×10^{-7} gm. per ml. in 0.1 M phosphate buffer at pH 7. The control was a solution containing 10^{-5} gm. virus per ml. The control virus solution and the enzyme-virus mixture in each test were inoculated on 12 half leaves of *Phaseolus vulgaris* var. Early Golden Cluster. In all cases the activity after standing was not significantly different from that of the solution when tested immediately after mixing. There was no suggestion, therefore, that the inactivation was due to a progressive hydrolysis of virus.

TABLE II
Effect of Dilution on Activity of Virus-Ribonuclease Mixtures

Virus-enzyme mixture (gm. protein per ml.)	<i>Phaseolus vulgaris</i> *				
	Dilution	No. of lesions		Concentration of control (gm. per ml.)	Per cent of original activity
		Mixture	Control		
Virus 10^{-3} Enzyme 1.2×10^{-7}	Undiluted	948	496	10^{-5}	1.9
	1-5	987	660	10^{-5}	7.5
	1-10	993	430	10^{-5}	23
	1-50	370	530	10^{-5}	35
	1-100	645	897	10^{-5}	70
	1-500	212	180	2×10^{-5}	120
	1-1000	47	51	10^{-5}	97
	Undiluted	39	53	10^{-5}	0.07
Virus 10^{-3} Enzyme 1.2×10^{-6}	1-5	128	33	10^{-5}	2.0
	1-10	186	40	10^{-5}	4.6
	1-50	233	43	10^{-5}	27
	1-100	160	71	10^{-5}	22
	1-500	139	109	10^{-5}	64
	1-1000	120	87	10^{-5}	138

* 12 to 16 half leaves were used for each mixture and its control solution.

† Calculated as in Table I.

Effect of Dilution and the Reversal of Inactivation.—It is well known that the dilution of inactive antigen-antibody complexes in many cases causes a partial reactivation of the antigen. If the observed inactivation of tobacco mosaic were due to an unspecific combination of virus with ribonuclease or to an inhibitory or toxic effect on the plant, then reactivation of inactive mixtures might also take place with dilution. The activities of several virus-enzyme mixtures were therefore compared at various dilutions with control solutions containing the same amounts of virus activity in the absence of enzyme. These results are summarized in Table II. It may be seen that a mixture of enzyme and virus which contained only a fraction of 1 per cent of the original virus activity was fully active when the solution was diluted from 1 to 500 to 1 to 1000 times.

It seemed apparent from these experiments that if a virus-enzyme complex were formed, it was readily dissociated. Attempts were made to restore the original specific activity of an inactive virus-enzyme mixture by repeated differential ultracentrifugation. Free or combined virus would be expected to sediment in a centrifugal field which would leave the uncombined enzyme in the supernatant liquid. Re-solution of the pellet in dilute phosphate buffer and sedimentation would leave an additional portion of enzyme in the supernatant liquid and repetition of the process should finally result in the elimination of all the enzyme.

TABLE III

Recovery of Virus Activity from an Inactive Virus-Enzyme Mixture by Repeated High Speed Centrifugation

Treatment	No. of lesions on <i>Phaseolus vulgaris</i> and activity					
	Experiment 1			Experiment 2		
	Mixture	Control	Per cent of original activity	Mixture	Control	Per cent of original activity
(1) 10^{-3} gm. virus + 2.7×10^{-4} gm. ribonuclease per ml.	0	2343	0			
(2) Above mixture centrifuged and sediment suspended in 0.1 M phosphate	127	756	17	273	1020	27
(3) Solution of sedimented virus from (2) treated as in (2)	1193	1761	68	635	770	82
(4) Solution of sedimented virus from (3) treated as in (2)	487	670	73	476	676	70
(5) Solution of sedimented virus from (4) treated as in (2)	198	325	61	680	741	92

*No. of lesions (mixture)
No. of lesions (control) $\times 100$.

Experimental Procedure.—0.21 ml. of ribonuclease solution containing 2.7×10^{-4} gm. ribonuclease per ml. was added to 14 ml. of purified tobacco mosaic virus containing 1 mg. of virus per ml. in 0.1 M phosphate buffer. This solution and the control containing an equal amount of virus were centrifuged in stainless steel tubes at 550 R.P.M. for $1\frac{1}{2}$ hours and the supernatant liquids were poured off. 10 ml. of 0.1 M phosphate at pH 7 were added to each tube and the virus pellets were redissolved. 0.1 ml. samples were removed from each tube, diluted with 9.9 ml. of 0.1 M phosphate at pH 7, and inoculated by the half-leaf method on 12 to 16 leaves of *Phaseolus vulgaris* var. Early Golden Cluster. The remaining solution was subjected to three additional centrifugal cycles, samples being removed as described above after each sedimentation.

After one sedimentation the specific activity of the sedimented material as shown in Table III was from 17 to 27 per cent that of untreated virus.

After two sedimentations the activity was about 70 per cent and after four sedimentations the activity of the treated virus was about the same as that of the original sample.

Crystallization and Analysis of Virus-Enzyme Complex.—When an enzyme solution was added to a solution of virus containing 10^{-3} gm. per ml. or more, in 0.1 *M* phosphate buffer at pH 7, it was observed that a precipitate formed which redissolved when the solution was mixed. When a dialyzed enzyme solution was added to a virus solution in the absence of salt, however, the precipitate which separated failed to redissolve. Microscopic examination

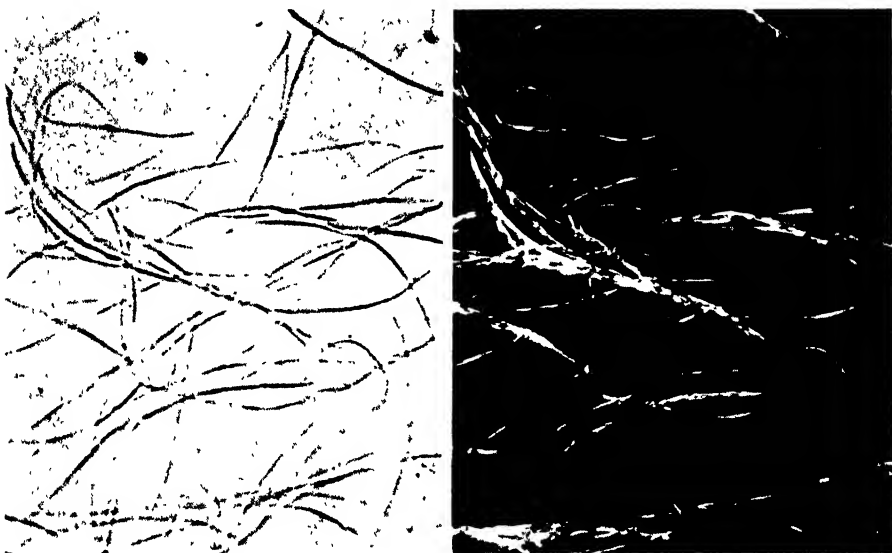


FIG. 2. Fiber crystals of tobacco mosaic virus-ribonuclease complex. 240 \times . Photographed in light and dark fields respectively.

showed the precipitate to consist of needles similar to those obtained when the purified virus is treated with ammonium sulfate. The careful addition of enzyme to a stirred-virus solution in the absence of salt resulted in the separation of long fibers as shown in Fig. 2. Nitrogen analyses of the supernatant liquids obtained after successive additions of enzyme showed that the amount of soluble nitrogen decreased to a minimum which was almost zero and then as more enzyme was added the value increased. The precipitate was insoluble in distilled water even after four washings in the centrifuge but could be dissolved readily by the addition of a trace of acid or alkali or phosphate buffer at pH 7.

In order to determine whether or not the precipitate consisted of virus alone or of virus in combination with enzyme or whether phosphorus had

been split off, nitrogen and phosphorus determinations were run on the washed precipitate and on the supernatant liquid and washings from a known quantity of virus and enzyme.

Experimental Procedure.—In Experiment 1, 0.2 ml. of an aqueous ribonuclease solution containing 2.84 mg. of ribonuclease nitrogen per ml. was added slowly with stirring to 10 ml. of tobacco mosaic virus solution containing 2.09 mg. of virus nitrogen. The resulting precipitate was centrifuged at 2200 R.P.M., the supernatant liquid was carefully removed with a dropping pipette, and the precipitate was washed four times in the centrifuge with 2 ml. portions of distilled water. The first supernatant liquid and the washings were combined, and the insoluble precipitate resuspended in water was dissolved by the addition of a drop of 0.1 N NaOH. Aliquots of both solutions were then taken for nitrogen and phosphorus analyses. In Experiment 2,

TABLE IV

Distribution of Nitrogen and Phosphorus in Precipitate and Supernatant Liquid after Mixing Known Quantities of Virus and Ribonuclease

2.09 mg. of virus nitrogen + 0.568 mg. ribonuclease nitrogen

Experiment	Insoluble precipitate		Supernatant liquid and washings		Recovery	
	Nitrogen*	Phosphorus*	Nitrogen	Phosphorus	Nitrogen	Phosphorus
	mg.	mg.	mg.	mg.	per cent	per cent
1	2.43	0.077	0.27	<0.001	102	103
2	2.40	0.074	0.26	<0.001	100	99

* Nitrogen and phosphorus determined by the methods of Levy and Palmer (10) and King (11) respectively.

the same procedure was followed with the exception that the ribonuclease added was dissolved in 2 ml. of water.

The results of two experiments are shown in Table IV. Because all the phosphorus was present as virus and was recovered in the precipitate, it can be concluded that the virus was quantitatively precipitated and that no nucleic acid was split off. A comparison of the nitrogen to phosphorus ratio of the precipitate with that of the original virus showed some additional enzyme nitrogen was also precipitated and was present in sufficiently strong combination to remain with the virus even after the precipitate had been washed four times.

The fact that a relatively insoluble virus-enzyme complex can be prepared provides evidence that the loss of virus activity when enzyme is added to virus in the absence of salt is due at least in part to the formation of such a complex. A comparison of the relative isoelectric points of the virus, pH 3.5,

and the enzyme about pH 8, shows that in a neutral solution such a complex would, in fact, be expected.²

DISCUSSION

The fact that no nucleic acid fraction is split off when ribonuclease is added to the active virus provides some insight into the type of combination between the protein of the virus and its nucleic acid and the nature of ribonuclease activity. Nucleic acid would appear to occupy an integral rather than a terminal position in the virus particle, for in the latter case hydrolysis by ribonuclease might be expected. Other evidence for such a hypothesis is the fact that the liberation of nucleic acid by treatment with alkali or urea is accomplished only with the destruction of the large virus particle into much smaller protein and nucleic acid components, as shown by the low rates of sedimentation of the split products (12, 13). In so far as ribonuclease activity is concerned it is evident that in the case of the tobacco mosaic virus nucleoprotein, ribonuclease fails to hydrolyze the linkage between nucleic acid and protein.

The inactivation with ribonuclease appears to be comparable to that found for other proteins with high isoelectric points, and is probably related to the formation of complexes between oppositely charged protein particles. The complex between virus and ribonuclease appears to be of the same type as that reported by Bawden and Pirie (14) to result from the action of clupein and papain on tobacco mosaic virus and as the ovalbumin-nucleic acid complex described by MacInnes and Longworth (15). The complex formed with tobacco mosaic virus unlike the latter, however, is insoluble in distilled water and gives the characteristic crystals or fibers. The solubility in salt or slightly acid or alkaline solutions suggests either partial or complete dissociation under these conditions as is the case for the ovalbumin-nucleic acid complex.

SUMMARY

The reversible inactivation of tobacco mosaic virus by crystalline ribonuclease is reported. Studies on the effect of time of standing on the amount of inactivation, and on the effect of dilution and repeated high speed centrifugation on the recovery of virus activity, and the preparation of an insoluble virus-enzyme complex show that the inactivation is brought about at least in part by a combination between virus and enzyme. The significance of the fact that ribonuclease has no detectable effect on the virus nucleic acid when the latter is in combination with protein in the form of virus is discussed with respect to the structure of the virus.

² A similar insoluble complex was formed with the bushy stunt virus and ribonuclease but in this case the precipitate was not definitely crystalline.

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THE COMPLEX NATURE OF WHITE-CLOVER MOSAIC

By FOLKE JOHNSON¹

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

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INTRODUCTION

White-clover mosaic, a disease that causes streak when transmitted to the garden pea (*Pisum sativum* L.) (13, 14, 24, 25), has been regarded as induced by a single virus, classified by Weiss (18) as *Trifolium virus* 1. In a recent study (7) on the transmission of viruses by dodder (*Cuscuta campestris* Yunck.), the writer isolated two distinct entities from white clover plants (*Trifolium repens* L.) affected by mosaic. Separation of the two viruses was possible because dodder transmitted only one of them, and the cowpea (*Vigna sinensis* (L.) Endl.) was susceptible only to the other. The virus transmitted by dodder will be referred to as pea-mottle virus and that isolated by means of cowpea will be designated as pea-wilt virus. The present paper presents the results of a study of the properties of these two viruses, their characteristic reactions, and their probable relationships with other legume viruses.

Literature Review

The literature reveals the fact that symptoms of streak in pea may be caused by any one of a number of distinct viruses or virus complexes. Linford (9) noted that pea streak was present in fields from the Atlantic coast to Utah and Montana. He subsequently showed (10) that in Hawaii a similar disease was produced in peas by the virus of pineapple yellow spot, now known to be identical (12, 15) with tomato spotted-wilt virus (*Lethum australiense* H.).² Linford's results were confirmed by Whipple (19) and Snyder and Thomas (16), who reported that spotted-wilt virus caused streak in garden peas and sweet peas. Adam (1), in South Australia, obtained results identical with those of Whipple. In a recent communication, Whipple and Walker (20) described two viruses believed to be strains of the common cucumber-mosaic virus (*Marmor cucumeris* H.), both of which caused streak in certain field-grown peas in Wisconsin. Further evidence that strains of the cucumber-mosaic virus were widespread in nature and caused streak in garden peas was

¹ At present, holder of the Elizabeth Clay Howald Scholarship, Department of Botany, Ohio State University, Columbus, Ohio.

² The Latin names used in this paper follow the system of nomenclature presented in the Handbook of Phytopathogenic Viruses (5).

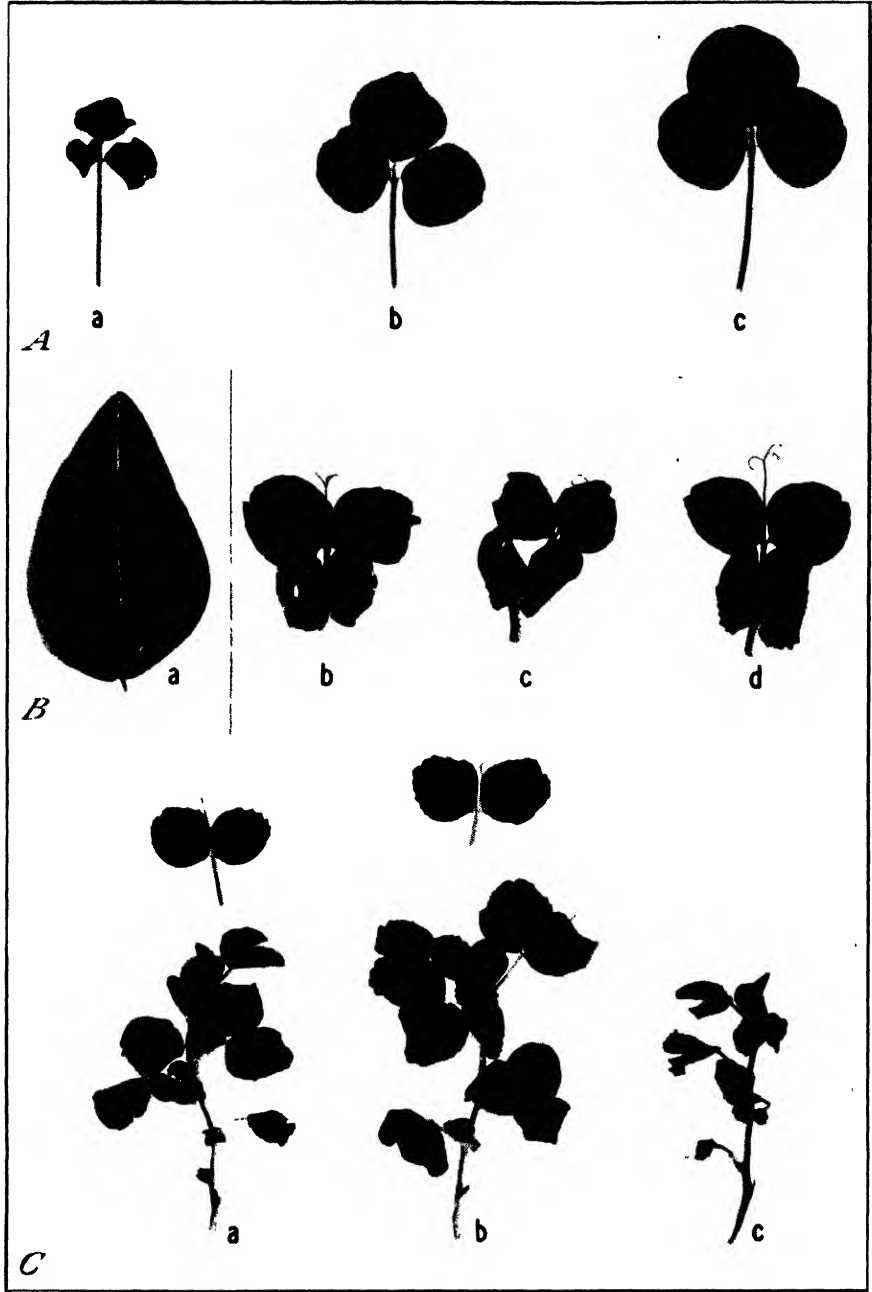


FIG. 1

given by Zaumeyer (23). Stubbs (17) reported that tobacco-ringspot virus (*Annulus tabaci* H.) caused streak and death of inoculated pea plants. Zaumeyer (22) described an outbreak of streak in garden peas exposed to pea aphids (*Macrosiphum pisi* Kalténbach) collected from field-grown alfalfa (*Medicago sativa* L.) and believed the disease to be caused by a distinct virus, which he called pea-streak virus 1; Zaumeyer also isolated two strains of alfalfa-mosaic virus (*Marmor medicaginis* H.), which gave symptoms nearly comparable to streak when used to infect peas. A systemic streak in greenhouse-grown peas inoculated with juice from mosaic-infected white clover was observed by Zaumeyer and Wade (24, 26) and by Pierce (13, 14); while Osborn (11) noted a similar disease caused by the red clover vein-mosaic virus (*M. trifolii* H.). Chamberlain (4) reported a new virus that caused pea streak in New Zealand, and more recently Ainsworth and Ogilvie (2, 3), showed that lettuce-mosaic virus (*M. lactucae* H.) caused streak and death of sweet peas in Great Britain. A single mention of pea streak occurring in China was made by Yu (21). Thus it is known that in addition to the reports of pea streak made by Chamberlain (4) and Yu (21), the viruses of tomato spotted-wilt (pineapple yellow-spot), cucumber mosaic, pea streak, alfalfa mosaic, white-clover mosaic, red-clover vein mosaic and lettuce mosaic caused streak and death to garden peas and sweet peas in various parts of the world.

Separation of the Viruses from the Complex

Pea-mottle virus was isolated by the following method: 12 white-clover plants infected with the virus complex (*Trifolium virus* 1) were joined by means of dodder to 12 healthy broad-bean plants (*Vicia faba* L.). Similarly, 8 hop-clover plants (*Medicago lupulina* L.), infected with the virus complex, were connected to 8 healthy hop-clover plants. Nine broad-bean and 5 hop-clover plants became diseased with mosaic during a period of time between 32 and 40 days after connecting them with the diseased plants. The hop-clover plants were mildly mottled and less stunted than those to which they were connected (Fig. 1, A). Inoculation of Dwarf Telephone peas with juice from the diseased broad beans and mildly affected hop clover produced symptoms of mosaic

FIG. 1. Symptoms produced by virus complex and separated virus constituents in several plants. A, a. Dwarfing and malformation in hop clover caused by virus complex (*Trifolium virus* 1) from mosaic-infected white clover. b. Mild mottling in hop clover produced by pea-mottle virus. c. Healthy hop-clover leaf. B, a. Local necrotic lesions in cowpea produced by pea-wilt virus. b, c. Mosaic in Dwarf Telephone pea caused by pea-mottle virus. Early symptoms at b, later symptoms at c. d. Healthy Dwarf Telephone pea leaves. C, a. Death of inoculated leaf resulting in Dwarf Telephone pea from infection of pea-wilt virus. b. Healthy Dwarf Telephone pea plant. c. Streak in Dwarf Telephone pea produced by infection with a combination of pea-mottle virus and pea-wilt virus. (Photographs by Julian A. Carlile.)

TABLE 1

Comparative Suscept Range of Pea-Mottle Virus and Pea-Wilt Virus According to Families

Host plant	Pea-mottle virus		Pea-wilt virus	
	Plants tested ^a	Symptoms ^b	Plants tested	Symptoms
Caryophyllaceae				
<i>Stellaria media</i> (L.) Cyrill.....	8/8	M	8/0	
Chenopodiaceae				
<i>Beta vulgaris</i> L. (Sugar beet)	10/0		10/0	
<i>Spinacia oleracea</i> L.....	5/5	M	5/0	
Compositae				
<i>Callistephus chinensis</i> Nees.....	10/0		10/0	
<i>Lactuca sativa</i> L.....	10/0		10/0	
<i>Taraxacum officinale</i> Weber.....	1/0		1/0	
<i>Zinnia elegans</i> Jacq.....	20/0		22/0	
Cruciferae				
<i>Barbarea vulgaris</i> R. Br.....	1/0		1/0	
<i>Brassica oleracea</i> L.				
var. <i>capitata</i> DC.....	5/0		5/0	
<i>Raphanus sativus</i> L.	5/0		5/0	
Cucurbitaceae				
<i>Cucumis sativus</i> L.....	25/24	YL	25/0	
Gramineae				
<i>Zea mays</i> L.....	16/0		19/0	
Leguminosae				
<i>Glycine max</i> Merr....	18/0		22/0	
<i>Lathyrus odoratus</i> , L.				
var. Bridal Veil	8/8	M	6/6	m, LN
var. Cardinal.....	7/7	M	7/7	m, LN
var. Treasure Island.....	8/8	M	9/4	m, LN
<i>Lens esculenta</i> Moench.....	16/16	VC, s	17/17	m, S
<i>Lupinus albus</i> L.	10/10	M, NS	10/1	VC
<i>L. hirsutus</i> L.....	10/10	M, NS	10/0	
<i>Medicago lupulina</i> L.	10/10	M	10/10	m
<i>M. sativa</i> L.....	10/6	M	10/0	
<i>Melilotus alba</i> Desr.....	18/18	M	18/8	m
<i>Phaseolus aureus</i> Roxb.....	15/1	VC	14/13	RS, NS
<i>P. vulgaris</i> L.				
var. Early Golden Cluster.....	15/15	M	15/6	m
var. Great Northern U. of Idaho				
No. 1.....	12/10	m	11/1	m
var. Ideal Market.....	10/0		11/0	
var. Kentucky Wonder.....	8/8	m	7/0	
var. Navy Robust.....	7/7	YL, m	9/0	
var. Red Kidney.....	8/8	YL, m	9/6	m
var. Red Valentine.....	16/16	M	11/11	m
var. Robust	9/9	YL, m	9/9	m
var. Stringless Refugee.....	9/9	M	9/5	m

TABLE 1—Continued

Host plant	Pea-mottle virus		Pea-wilt virus	
	Plants tested ^a	Symptoms ^b	Plants tested	Symptoms
Leguminosae				
<i>P. vulgaris</i> L. (cont.)				
var. Stringless Refugee Green Pod	9/9	M	9/4	m
var. U. S. No. 5 Refugee	12/9	YL, m	12/8	m
var. Unrivalled Wax	12/12	YL, m	12/6	m
<i>Pisum sativum</i> L.				
var. Alaska	15/10	M	17/15	m, LN
var. Dwarf Alderman	30/28	M	30/30	LN
var. Dwarf Telephone	28/26	M	30/30	LN
var. Hundredfold	39/38	M	36/35	LN
var. Laxton Progress	26/20	M	23/23	LN
var. Little Marvel	16/16	M	18/18	LN
var. Nott's Excelsior	18/18	M	20/20	LN
var. Perfection	23/23	M	29/29	LN
var. Potlatch	21/21	M	18/18	LN
var. <i>arvense</i> Poir. Canada White	20/20	M	19/18	m, LN
<i>Trifolium hybridum</i> L.	5/5	M	5/5	None
<i>T. incarnatum</i> L.	10/10	m, VC	10/10	VC
<i>T. pratense</i> L.	10/10	M	10/10	m, VC
<i>T. repens</i> L.	10/10	M	10/10	M
<i>Vicia faba</i> L.	25/18	M, ns	23/13	m, RS
<i>V. sativa</i> L.	16/16	m, NS	16/16	m, VC
<i>Vigna sinensis</i> (L.) Endl.	30/0		50/38	m, BLL
Liliaceae				
<i>Lilium formosanum</i> Stapf.	10/0		10/0	
Plantaginaceae				
<i>Plantago lanceolata</i> L.	1/0		1/0	
<i>P. major</i> L.	1/0		1/0	
Polygonaceae				
<i>Rumex acetosella</i> L.	1/0		1/0	
Scrophulariaceae				
<i>Antirrhinum majus</i> L.				
var. Giant Crimson	8/5	M	8/0	
var. Giant White	8/3	M	8/0	
Solanaceae				
<i>Datura stramonium</i> L.	5/0		5/0	
<i>Lycopersicon esculentum</i> Mill.	15/0		15/0	
<i>Nicotiana glutinosa</i> L.	35/0		40/0	
<i>N. tabacum</i> L.	35/0		45/0	
<i>N. rustica</i> L.	5/0		5/0	
<i>N. sylvestris</i> Spegaz. and Comes	20/0		20/0	
<i>Solanum nigrum</i> L.	5/0		5/0	

^a The numerator indicates the number of plants inoculated, denominator indicates the number of plants diseased.

^b M = mottling; S = streak; YL = systemic yellow lesions; NS = necrotic spotting; VC = vein clearing; RS = ring spotting; LN = basal leaf wilting and necrosis; BLL = brown local lesions. Similar descriptions with small letters indicate these symptoms were mild.

(Fig. 1, *B*, *b*, *c*) rather than those of streak. The same result was obtained when pea plants were inoculated with juice extracted from dodder that had parasitized infected white clover. On the other hand, inoculations of similar plants with juice from the diseased white clover invariably resulted in streak. The results led to the belief that the white clover was infected with more than one virus, and that dodder had isolated but one constituent of the complex, namely pea-mottle virus.

The second constituent of the complex, referred to as pea-wilt virus, was isolated by inoculation of cowpea with juice from the diseased white clover, since it was found that cowpea plants were not susceptible to infection with pea-mottle virus. Cowpea leaves inoculated with plant juice containing the virus complex developed brown, necrotic, local lesions (Fig. 1, *B*, *a*). On transfer from infected cowpeas to Hundredfold peas, the virus produced no definite local lesions, but caused wilting and death of the inoculated leaves (Fig. 1, *C*, *a*). The infected pea plants did not develop streak or chlorotic mottling, but showed only a mild discoloration of the stem; nevertheless, it was easy to demonstrate the presence of virus in the tops of infected plants by inoculation of expressed juice to cowpeas. The pea-wilt virus isolated in this manner, when mixed with the previously isolated pea-mottle virus, caused typical symptoms of streak in Dwarf Telephone and Hundredfold peas (Fig. 1, *C*, *c*).

Suscept Range

In order to obtain a better understanding of the relationship between the two viruses, a knowledge of their suscept ranges was needed. An attempt was made to test as far as possible those species and varieties of plants used by other investigators in their work with legume viruses. All plants were grown from seed in a greenhouse held at about 25° C. and fumigated regularly to destroy insects. Plants tested for susceptibility were young and in a stage of rapid growth. The test plants were dusted with carborundum powder and inoculated by the rubbing method. Inoculum was prepared from diseased tissue of young, rapidly growing plants by macerating it in a sterile mortar to which a few drops of tap water were added. A sterile cotton swab on a small stick was dipped in the inoculum and gently rubbed over the plant tissue, which was supported with a sterile pot label. This method of inoculation has been described in detail by Jones (8). When plants with large leaves were inoculated, sterile gauze pads were used instead of the cotton swabs, and the leaves were supported in the hand. Immediately after inoculation the plants were rinsed with water from a sprinkling can in order to remove any toxic materials. From 2 to 5 leaves were inoculated, depending upon the size and growth habit of the plants. After a suitable incubation period, sub-inoculations were made to peas with juice from the tested host plants.

As is shown in table 1, pea-mottle virus infected plants in the Caryophyllaceae, Chenopodiaceae, Cucurbitaceae, Scrophulariaceae, and Leguminosae, while pea-wilt virus infected plants of the Leguminosae only.

Symptomatology

Pea-mottle Virus. When pea plants were inoculated with pea-mottle virus, the developing leaves failed to open as readily as in healthy plants; and from 8 to 12 days after inoculation a fine clearing of veins appeared in the young foliage. The large veins were bleached and the network of fine veins stood out in contrast to the adjacent tissues (Fig. 1, B, b). Numerous, small, irregular, light-yellow spots were scattered over the youngest foliage. Infected plants were slightly stunted and lighter in color than healthy plants. The first two leaves that developed after the mottling was noticeable were more severely affected than the succeeding ones in which the yellow spots coalesced to form large light-green areas (Fig. 1, B, c). The stipules showed the same type of mottle that was found in the leaves. Some varieties of plants outgrew the mottling before blooming. No symptoms were noticeable on the stems; likewise, no apparent effects were observed in the pods or seeds of infected plants.

On bean (*Phaseolus vulgaris* L.), pea-mottle virus produced light-yellow spots and clearing of veins (Fig. 2, A, a). These symptoms were uniform on the varieties that were tested. On Alsike clover (*Trifolium hybridum* L.), red clover (*T. pratense* L.) and white clover, light-yellow areas appeared between the veins. The symptoms in alfalfa were distinct, consisting of irregular streaks of yellowing along the veins and adjacent tissues. In some cases dark-green irregular patches of tissue became outlined with light-yellow margins. Infected spinach plants (*Spinacia oleracea* L.) became dwarfed and severely mottled, and infected cucumber (*Cucumis sativus* L.) showed light-yellow secondary lesions (Fig. 2, B, b, c).

Pea-wilt Virus. Symptoms produced by this virus in pea were noticeable in from 5 to 8 days following inoculation. The inoculated leaves wilted and died and the petioles shriveled, leaving the dead, dried leaves attached to the stem (Fig. 1, C, a). One or more of the adjacent, lower leaves also wilted and died. In most cases the tops of the plants appeared healthy, but in two varieties, Alaska and Canada White, a faint mottling developed and soon disappeared. The stems showed a faint grayish discoloration. Infected plants grew slowly and were dwarfed in comparison with healthy plants.

In cowpea plants the inoculated primary leaves developed brown, local lesions (Fig. 1, B, a), while the trifoliate leaves showed isolated diffuse areas where the veins became slightly bleached. In mung bean (*Phaseolus aureus* Roxb.), necrotic zonate lesions were produced on the inoculated primary leaves (Fig. 2, C, b), and in some instances dark necrotic spots developed in systemically invaded leaves. A blotchy mosaic disease was produced in

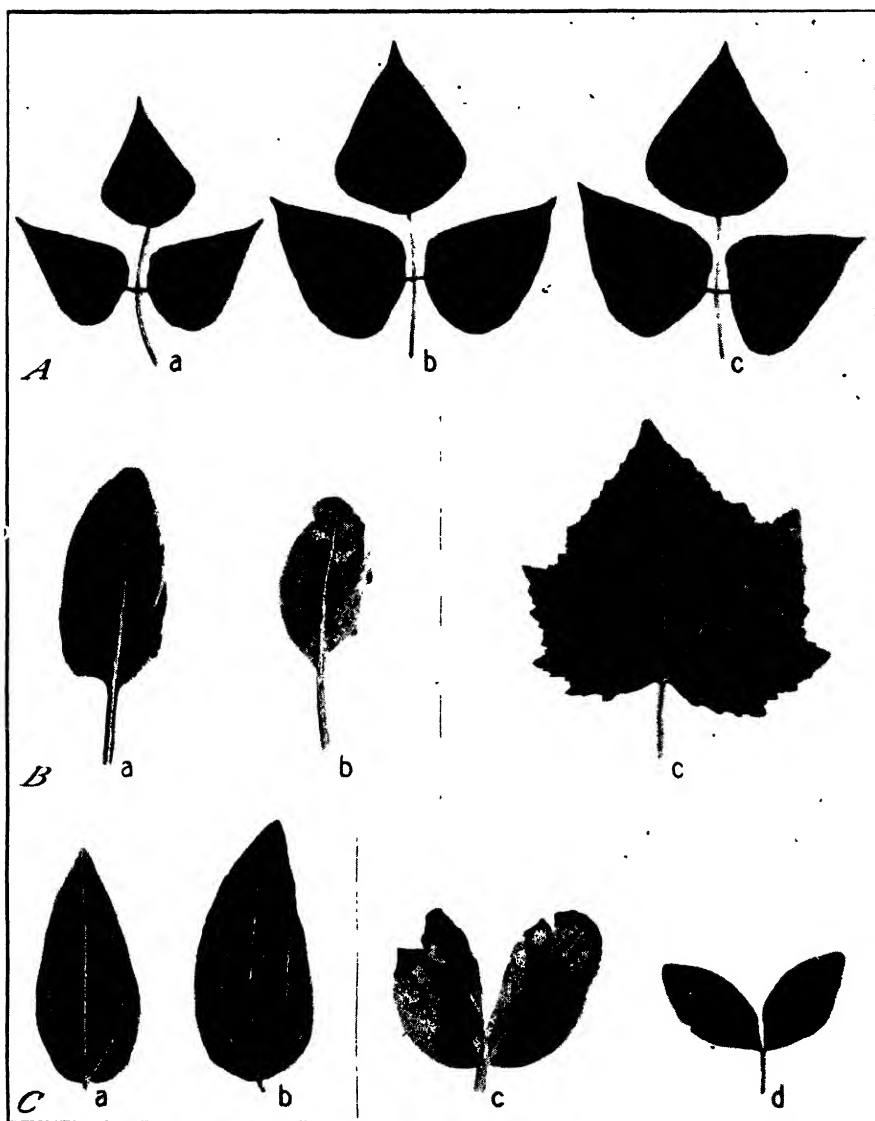


FIG. 2. Symptoms produced by separate virus constituents in several plants. *A*, a. Symptoms in Red Valentine bean caused by pea-mottle virus. b. Healthy Red Valentine bean. c. Diffuse mottle in Red Valentine bean caused by pea-wilt virus. *B*, a. Healthy spinach leaf. b. Mottling in spinach caused by pea-mottle virus. c. Symptoms in cucumber caused by pea-mottle virus. *C*, a. Healthy *Phaseolus aureus* leaf. b. Zonate necrotic spots in inoculated leaf of *P. aureus* caused by pea-wilt virus. c. Ring spotting and necrosis in broad bean caused by pea-wilt virus. d. Mosaic in broad bean caused by pea-mottle virus. (Photographs by Julian A. Carlile.)

infected bean plants (Fig. 2, A, c). Most of the clovers that were infected showed only a very faint mottle, but white clover was noticeably mottled. Alsike clover showed no symptoms of disease, although the plants were systemically invaded by the virus.

Histopathology

Epidermal strippings and cross sections of stems of Alsike clover, red clover, white clover, hop clover, and several varieties of pea infected with either pea-mottle virus or pea-wilt virus were examined under the microscope for intracellular inclusions. In each case the diseased material was compared with healthy tissue. Water and several other mounting media were used, but iodine-potassium iodide solution, in the proportion of 1 part iodine, 3 parts potassium iodide, and 150 parts water, gave the best results, staining the nuclei brown. Examinations failed to reveal inclusion bodies or unusual crystalline deposits. Calcium oxylate crystals were found in both healthy and diseased plants, but plate crystals, like those found in tobacco and other plants infected with certain viruses, were not observed.

Transmission Studies

In addition to the transmission experiments with dodder, an attempt was made to determine whether or not the pea aphid (*Macrosiphum pisi* Kalt.) was a vector of these viruses under laboratory conditions. Plants of red clover, white clover, hop clover, and Dwarf Alderman peas infected with each virus were caged separately and 30 healthy aphids added to each cage. Healthy plants of each species were treated similarly and used as controls. After a feeding period of 4 days on the diseased and healthy control plants, the aphids were transferred separately from each cage to 6 healthy, caged Dwarf Alderman peas. An attempt was made to place an equal number of insects on each pea plant. The aphids were allowed to feed for a period of 4 days after which they were killed by fumigation, and the plants removed to a greenhouse bench for observation. No symptoms of disease appeared on any of the test plants; likewise, all control plants remained healthy. Since pea-wilt virus did not produce mottling in pea, it seemed possible that the pea plants on which the aphids fed might have been infected without showing definite symptoms. To determine whether this was actually the case, a small portion of leaf tissue was removed from each plant, macerated in a mortar to which pea-mottle virus was added, and used as inoculum for healthy peas. Typical symptoms of pea-mottle developed in the inoculated plants instead of those of streak which would have been the result had the plants been infected with both viruses. This result demonstrated that pea-wilt virus had not been transmitted to peas by the pea aphid.

A second test was made in the following manner: White clover plants

infected with each virus were caged separately. One hundred healthy aphids were placed in each cage and allowed to feed 2 days. The aphids from each cage were then transferred separately to 25 individually caged, healthy Dwarf Telephone peas, 4 insects being placed on each plant. All insects were killed after a feeding period of 11 days and the plants removed to a greenhouse for observation. No symptoms of disease developed. As a further check on these plants, a portion of leaf tissue was removed from each plant of each lot, macerated together, and tested for virus by mechanical inoculation of healthy peas. These tests also were negative. The possibility existed that pea-wilt virus was present without showing symptoms of disease; in this event no stream would have appeared if pea-mottle virus had been absent. To test this possibility, leaf tissues from each pea plant in the pea-wilt-virus series were macerated together and pea-mottle virus was added to the plant juice. This mixture was used to inoculate healthy peas. Symptoms of pea-mottle developed in the inoculated plants instead of streak, which would have resulted if both viruses had been present in the inoculum. It is concluded from these results that the pea aphid was not a vector of these viruses.

Properties of the Viruses

Thermal Inactivation. Plants infected with each virus were ground separately in a sterile meat chopper, and the juice was extracted by passage through two layers of cheesecloth. The juice was placed in tightly stoppered test tubes (7 × 70 mm.) and completely immersed for 10 minutes in an electrically heated water bath. The water was stirred constantly by means of a stirring rod attached to an electric motor and the temperature was automatically controlled within $\pm 0.2^{\circ}\text{C}$. of the desired temperature. After a 10-minute immersion the test tubes were immediately plunged into ice water, and the cooled cell extract was used to inoculate healthy plants.

Infected plants of Dwarf Alderman, Dwarf Telephone, and Potlatch pea served as source plants for the viruses, and healthy plants of the same varieties were used as test plants. Between 15 and 20 plants were inoculated with each sample and each test was conducted on 3 different occasions. Attempts were also made to use cowpea and mung beans as test plants for measuring activity of the pea-wilt virus, but with erratic results. Therefore, in order to test for pea-wilt virus in heated samples, a small amount of pea-mottle virus was added to each sample after it had cooled. The test plants reacted by production of mottling symptoms when only the pea-mottle virus was present, by production of streak when pea-wilt virus was also present. It was found that pea-mottle virus was inactivated by exposure for 10 minutes to a temperature of $60\text{--}62^{\circ}\text{C}$., whereas pea-wilt virus was inactivated by exposure to a temperature of $58\text{--}60^{\circ}\text{C}$.

Tolerance to Dilution. Infectious plant juice was extracted, as previously

reported, and diluted with distilled water in varying proportions up to 1 part in a million. Fifteen to 20 Dwarf Telephone peas were inoculated with each diluted sample. Pea-mottle virus was still infectious at a dilution of 1:10,000, but not 1:100,000, while pea-wilt virus was recovered at a dilution of 1:100,000 but not 1:1,000,000.

Resistance to Aging. Expressed plant juice from plants infected with each virus was stored separately in stoppered bottles at room temperature (about 25° C.) and used as inoculum for 18 to 20 Dwarf Telephone peas. Inoculations were made at 3-day intervals for 3 successive times; subsequent inoculations were made at irregular intervals up to 31 days after extraction. Both viruses withstood aging *in vitro* for 31 days. Neither virus was tested for resistance to aging for longer than this period.

Pea plants infected with pea-mottle virus, pea-wilt virus, and broad bean leaves infected with Zaumeyer and Wade's pea virus 2 were dried at room temperature, held in separate stoppered bottles, and tested for their resis-

TABLE 2
Comparison of Physical Properties of Pea-Mottle Virus and Pea-Wilt Virus

Virus	Thermal inactivation	Tolerance to dilution	Resistance to aging		Filterability
			in vitro	in dry host tissue	
Pea-mottle virus	60-62° C.	1:10,000	At least 31 days	At least 31 days	+
Pea-wilt virus	58-60° C.	1:100,000	At least 31 days	At least 31 days	+

tance to aging in dried host tissue. The first inoculation with the dried material was made 7 days after the plants were cut, at which stage the plant tissue was thoroughly dry. A small amount of the dried material was soaked with a few drops of tap water in a sterile mortar, macerated and the plant extract used as inoculum for either Dwarf Alderman or Dwarf Telephone peas. Inoculations were made at 3-day intervals for 3 successive times, after which they were made at irregular intervals. It was found that all the tested viruses withstood aging in dried plant tissue for at least 31 days.

Filterability. Pea plants infected with each virus were separately ground in a meat chopper and the juice was passed through 2 layers of cheesecloth, after which it was centrifuged for 15 minutes at 3300 R.P.M. The liquid was decanted and filtered through a layer of medium-size Celite before passage through a Berkefeld W filter. A 1 cc. sample of the filtered pea juice from each series of plants was added to laboratory broth and thus shown to be free from contamination; subsequently, portions of the filtered plant juice were used as inoculum for 20 to 25 Dwarf Telephone pea plants. In one test, infection was obtained in all plants inoculated with filtered pea-wilt juice, but no plants became diseased when inoculated with pea-mottle filtrate. In a

subsequent experiment it was shown that pea-mottle virus passed a Berkefeld W filter and infected inoculated plants. These results prove that both pea-wilt virus and pea-mottle virus pass Berkefeld W filters.

The physical properties of the two viruses are summarized in table 2.

DISCUSSION

The fact that *Trifolium virus 1* has proved to be a mixture of two viruses was not altogether unexpected, having been suggested by Zaumeyer and Wade (25), who found that the so-called *Trifolium virus 1* produced two types of symptoms on many bean varieties. In addition, they reported two different temperatures of virus inactivation and suggested that two viruses might be involved. They, however, did not separate the suspected constituents from the mixture. Pierce (13) found a mosaic infected red-clover plant in the field, the juice from which produced streak when transferred to pea. Inoculations of small-seeded broad bean with juice from the infected red clover resulted in local, necrotic lesions on inoculated leaves followed by a systemic mottling. When extract of the mottled tissue of broad bean was used to inoculate peas, only a mild mottling developed. Pierce considered the mottling virus to be identical with the one he previously had described and named bean virus 2, while the virus that produced local lesions in broad beans was named broad-bean local-lesion virus. Little is known about the latter virus and its reactions in pea. Pierce, however, suggested that when the mottling virus and broad-bean local-lesion virus were combined and used to inoculate pea, streak would be induced, but he cited no definite experimental evidence for this conclusion. It is believed that Pierce and the writer probably worked with the same virus complex, since the writer's original material was collected near Pullman, Washington, a few miles from the locality where Pierce found his infected red clover. This belief is supported by the fact that pea-mottle virus produces a faint mottle in broad beans, while pea-wilt virus causes the production of necrotic ring-spots in the same plants (Fig. 2, C, c, d). However, bean virus 2 of Pierce was reported to be non-infectious for white clover and alfalfa and also differed from pea-mottle virus in its resistance to aging *in vitro* and in its tolerance to dilution, thus suggesting that the two viruses are different. The writer believes that Pierce's evidence for considering that bean virus 2 was a component of his red clover virus complex was inconclusive, since it was based only on his observations of the symptoms produced in Stringless Refugee Green beans by his broad-bean mosaic virus. It is the writer's belief that Pierce had the virus complex in red clover that has been classified by Weiss (18) as *Trifolium virus 1*, but that is shown in this paper to consist of two separate viruses. Pea-mottle virus is similar to the virus that Pierce found to become systemic in broad bean and that produced a mottling in peas, whereas the pea-wilt virus of the writer is probably closely related to Pierce's (13) broad-bean local-lesion virus.

Pea virus 2 of Zaumeyer and Wade (25) resembles pea-mottle virus in the symptoms it produces on many pea and bean varieties. A further similarity between these two viruses is their resistance to drying in host tissue. Other similarities between the two viruses can be found in their susceptible range, although they differ somewhat in this respect. One point of difference between pea virus 2 and pea-mottle virus is the fact that pea virus 2 does not produce streak in peas when transferred to these plants in combination with the pea-wilt virus discussed in this paper.

Severe pea-mosaic virus previously described (6) resembles pea-mottle virus on the basis of susceptible range, longevity *in vitro* and dried tissue, tolerance to dilution, and heat inactivation. This suggests that these two viruses may be related.

Since it was shown that pea-mottle virus was infectious for plants in 5 different families, the question arises whether this virus is not related to one of the strains of cucumber-mosaic virus that have been found infecting peas (20, 23). Whipple and Walker (20) have shown that 2 cucumber viruses are infectious for a large number of plants in several plant families, including peas and other legumes. Pea-mottle virus has failed to infect corn (*Zea mays* L.), zinnia (*Zinnia elegans* Jacq.), or solanaceous plants, which are generally considered susceptible to infection with cucumber-mosaic virus. Likewise, the symptoms produced by the two viruses studied by Whipple and Walker in peas, beans, and cucumbers differ from those produced by pea-mottle virus.

Not only was it true that pea-mottle virus was infectious for plants in the Caryophyllaceae, Chenopodiaceae, Cucurbitaceae, Scrophulariaceae, and Leguminosae, whereas pea-wilt virus was infectious only for plants in the Leguminosae, but also, in all cases where plants were susceptible to both viruses, a more severe disease was produced by pea-mottle virus than by pea-wilt virus, except in the case of white clover, where similar symptoms were produced by both.

The evidence at hand points to the conclusion that the two viruses discussed in this paper are distinct from each other and that they show characteristics that distinguish them from any of the viruses previously given scientific names according to the system of nomenclature outlined by Holmes (5). In accordance with this system, pea-mottle virus may be referred to as *Marmor efficiens* n. sp., from Latin *efficiens* meaning effective, in reference to the ability of this virus to cause mottling in peas in contrast with the inability of pea-wilt virus to produce such chlorotic symptoms in tested varieties of this host other than Alaska and Canada White. Probable synonyms are: broad-bean-mosaic virus of Pierce (13), which was present in his red-clover-mosaic complex; severe pea-mosaic virus, previously described by Johnson and Jones (6); possibly also pea virus 2 of Zaumeyer and Wade (25). Pea-wilt virus may be referred to as *M. repens* n. sp. The specific name is taken from Latin *repens*

(not from *repens*) and means unlooked for, in reference to the unexpected discovery of a second virus in the original complex. Broad-bean local-lesion virus of Pierce (13) may be considered as a probable synonym.

SUMMARY

It has been shown that white-clover mosaic, a disease previously regarded as caused by a single virus classified as Trifolium virus 1, was actually induced by a mixture of two distinct viruses: namely, pea-mottle virus and pea-wilt virus. Their separation was accomplished because pea-mottle virus alone was transmitted by dodder (*Cuscuta campestris*), while pea-wilt virus infected cowpea (*Vigna sinensis*), a plant resistant to pea-mottle virus.

When pea-mottle virus in combination with pea-wilt virus was transferred to peas, streak was produced and resulted in death of the plants in a manner similar to that produced by the Trifolium virus 1 complex.

Pea-mottle virus alone produced a systemic mosaic disease when transferred to several pea varieties, and was infectious for plants in the Caryophyllaceae, Chenopodiaceae, Cucurbitaceae, Scrophulariaceae, and Leguminosae. In the tests conducted, pea-wilt virus was infectious for plants in the Leguminosae only, and produced no mottling in pea varieties, except Alaska and Canada White, where a very mild mosaic was produced. In general, plants infected with pea-mottle virus were more severely affected than when similar plants became infected with pea-wilt virus. No intracellular inclusion bodies could be detected in plants infected with either virus, and no virus transmission was obtained by allowing the pea aphid (*Macrosiphum pisi*) to feed on infected and healthy pea plants in succession.

Pea-mottle virus was inactivated by exposure for 10 minutes to a temperature of 60–62° C., whereas pea-wilt virus became inactive between 58–60° C. Pea-mottle virus was recovered in a dilution of 1:10,000 in water, and pea-wilt virus in a dilution of 1:100,000. Both viruses withstood aging *in vitro* and in dried host tissues for at least 31 days and were filterable through a Berkefeld W filter.

The name *Marmor efficiens* n. sp. is suggested for pea-mottle virus and *M. repens* n. sp. for pea-wilt virus.

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HETEROMORPHIC COLONIES ASSOCIATED WITH RING FORMATION

By K. W. KREITLOW

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for
Medical Research, Princeton, New Jersey)

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A striking phenomenon was observed in heavily seeded dilution plates of the bean-halo-blight organism, *Phytomonas medicaginis* (Sackett) Bergey *et al.* var. *phaseolicola* Burkholder. A convoluted variant of this species (plate I, fig. 1) characterized by colonies with rubbery consistency, rolled margins, and depressed centers was isolated from a sector occurring in a smooth colony. Broth cultures of the variant produced no colonies resembling the original smooth form, but gave rise to two distinct kinds of convoluted colonies on agar plates. Colonies of one form occupied an irregularly shaped circular area that varied in diameter from several centimeters to almost the width of the plate. The outer edge of this area consisted of colonies that were greatly enlarged so that a raised ring was formed. The other colony-form occupied the entire area outside the raised ring.

Colonies within the ring were translucent, semi-fluid, and lobed, as though by renewed growth from points on their peripheries. Those that made up the ring itself were translucent, semi-fluid, and greatly enlarged. Colonies outside the ring were white in color and rubbery in consistency.

Microscopically, the colonies inside the ring were translucent, colorless, and composed of elongated, thin, non-capsulated cells. Those outside the ring were opaque, light brown, and composed of short, plump, capsulated cells. Bacteria from all colonies reacted negatively to Gram's stain.

A study of the ring showed that, while it usually developed near the center of the plate, it sometimes occurred towards one side. In all cases it followed the curvature of the bottom of the plate, which controlled the thickness of the medium. The first noticeable sign of its formation was manifested after a 24- to 48-hour incubation by a swelling of the colonies on a certain area of the plate. The ring was usually well-formed in from 48 to 72 hours after the plate was poured. Typical rings are shown in plate I, figures 2 to 4.

A striking correlation was found between colony-form and ability of the bacteria to grow when transferred to fresh culture media. Organisms from the ring or the area within the ring initiated growth readily at any time when transferred to fresh media. Bacteria from outside the ring failed to grow in fresh media, no matter how long they were incubated.

This phenomenon differs from the concentric "ring formation" caused by

periodic swarming in species of the *Proteus* group. The *Proteus* phenomenon was first reported by Hauser (1885) and later studied by Moltke (1929), Russ-Münzer (1934-35), and Knöll (1939). Ring formation by *Phytomonas medicaginis* var. *phaseolicola* appeared to differ also from the "Bakterienniveaus" first reported for liquid media by Beijerinck in 1893, later studied by Jegunow (1896), Lehmann and Curchod (1905), and Eisenberg (1919), and more recently studied in solid media by Williams (1939). A search of the literature revealed no further references that might indicate a previous description of the phenomenon reported here.

The regular occurrence of heteromorphic colonies and their surprising correlation with viability stimulated further investigation to determine the conditions responsible for their origin and peculiar behavior.

Experimental Production of Rings

Experiments were conducted to test the effect of thickness of medium on ring formation. Difco nutrient agar to which 1 per cent glucose had been added was used throughout the studies to be reported. Except as otherwise stated, the medium was adjusted to neutrality and tubed in amounts of approximately 8 ml. per tube. Dilution series were made in sets of 3 nutrient-agar tubes by the following procedure: Three loopfuls of bacteria from a 48-hour broth culture were transferred to the first melted and cooled agar tube of each dilution series. Three loopfuls of material were then transferred successively from tube to tube through the series of 3 tubes. Each tube was rolled between the palms of the hands to insure thorough dispersion of the bacteria before a transfer was made to the next tube. Each dilution tube was then poured in a plate containing a layer of solidified nutrient agar. The plates were inverted for incubation at 28°C.

Bacteria in heavily seeded deep-agar plates behaved differently from those in ordinary poured plates. Ring formation either failed to occur or occurred only rarely. The colonies in deep agar appeared to break down and partially liquefy only at the edge of the plate where the medium had dried out and shrunk away from the glass. Colonies at the outer edge of the plate swelled and grew on the side walls where a thin film of medium adhered to the glass. Breakdown and liquefaction of the colonies was not so extensive as in ordinary plates and was confined to colonies immediately adjacent to the periphery of the plate.

As a further test of the hypothesis that thickness of the medium influenced ring formation, a number of watch glasses of different sizes were placed in various parts of Petri plates and sterilized. A layer of nutrient agar was then poured into the plates, so that all but a small portion of the top of each watch glass was immersed in agar, leaving a small rounded dome of glass projecting out of the medium. Dilution tubes were prepared as before and the watch

glasses completely covered, the medium being thinnest directly over the highest part of each watch glass. After suitable incubation, a ring formed around each watch glass and close examination showed that colonies within the ring broke down first over the highest part of each watch glass where the medium was thinnest.

The same type of experiment was repeated, using a sterile microscope slide in each Petri plate in place of a watch glass. The ring then followed the shape of the slide and colonies were found to break down over the surface of the medium above the slide where the agar was thinnest. The manner in which a ring followed the outline of a slide is shown in plate II, figure 5.

When agar dilution plates were poured and the plates tilted so that the medium solidified in a layer of progressively increasing thickness from one side of the plate to the other, no rings formed. Breakdown of the colonies occurred on that side of the plate where the medium was thinnest. The semi-fluid colonies were separated from the white, convoluted colonies in the thicker agar by a straight raised line. The same phenomenon was observed on agar slants. The breakdown of the colonies occurred at the upper end of each slant where the medium was thinnest. The swollen, cleared growth was sharply demarcated from the growth on the lower portion of the slant in the earlier stages of breakdown. Later, the semi-fluid character of the upper growth caused it to flow down over the rest of the slant, thus giving the entire slant a semi-fluid appearance.

All of these experiments demonstrated that ring formation was influenced appreciably by thinness of the medium.

Effect of H-Ion Concentration of the Medium on Viscosity of the Colonies and on Ring Formation

Difco nutrient agar plus 1 per cent glucose was adjusted with 0.1 N NaOH and HCl to pH 6.2, 7.6, 8.6, and 9.7. The medium was placed in tubes and used in dilution series to determine whether or not hydrogen-ion concentration would influence breakdown of the colonies. Dilution plates at the various pH's revealed that an alkaline reaction favored breakdown and that clear and sharply pronounced rings were produced only in plates at pH 6.2. Bacteria grown at pH 6.2 produced the typical white, convoluted colony-form in 48 to 72 hours with breakdown of colonies in the thinner portions of the plates. The colonies which developed at pH 7.6 retained their convoluted form but were translucent and partially fluid throughout their entire growth period. The colonies which developed at pH 8.6 and 9.7 were more hyaline and fluid as the alkalinity of the medium was increased.

Material from semi-fluid colonies grown on alkaline agar was plated in dilution series at pH 6.2. The resulting growth formed typical white, convoluted colonies and rings. These results showed that an acid reaction of the

culture medium favored the manner of growth conducive to colony breakdown and ring formation.

Changes in the H-Ion Concentration of the Medium on Which Rings Appeared

Heavily seeded Petri plates showing typical ring formation were tested for reaction of the medium by placing drops of bromthymol blue or cresol red indicator in various portions of each plate. Drops of bromthymol blue placed within a ring turned intensely blue, indicating an alkaline reaction of the medium. Drops of this indicator placed outside the ring became yellow, demonstrating acidity of the medium. Cresol red as an indicator likewise gave a red color within each ring and a change to yellow outside the ring. That ring formation had resulted in a change of reaction in the medium was further confirmed by placing strips of bromthymol blue or cresol red paper on the surface of the medium across the entire plate. Bromthymol blue paper turned intensely blue over the area within each ring. The ring itself gave a blue reaction at its inner margin and green at its outer edge. The part of the indicator paper extending from the outer edge of the ring to the walls of the Petri plates remained bright yellow in color. Figures 6 to 8 of plate II show the changes which took place in the indicator paper.

Dilution plates containing well-separated colonies that had broken down to a translucent, semi-fluid state also displayed an alkaline reaction of the medium in the immediate vicinity of each colony. On the other hand, white convoluted colonies that showed no signs of breaking down did not cause the medium to become more alkaline.

The exact nature of the material causing the alkaline change in the medium has not been determined. The white, convoluted colonies were, however, easily broken down by placing them in 0.1 N sodium or potassium hydroxide for a few minutes. The solution obtained was quite clear but viscid. Addition of a small quantity of acid caused the liquid to gel immediately into a turbid, slimy mass which was redissolved by addition of alkali. Nutrient broth adjusted to pH 7.8 to 8.0 was capable of dissolving the capsular material of the white, convoluted colonies overnight. Nutrient broth at a pH below 7.6 caused no dissolution of the capsular material.

Viability of the Colonies on Plates Showing Ring Formation

The relationship between ring formation and ability of the bacteria to grow on sub-transfer was studied in detail. Typical well-separated colonies first appeared hyaline, moist, and raised. They reached their maximum size after 48 to 72 hours, at which time they were white and convoluted with raised margins and depressed centers. If ring formation took place, colonies at and within the ring remained hyaline and did not mature into the convoluted form; only those outside the ring became white and convoluted. When the

colonies outside each ring were streaked on agar slants or placed in nutrient glucose broth, no growth took place even after prolonged incubation. On the other hand, when the partially broken-down colonies within a ring were transferred to agar slants or broth, growth readily took place, giving rise once more to the typical convoluted form. Bacteria that made up the ring itself likewise grew readily on agar or in broth.

Heavily seeded plates were incubated for 24 hours, at the end of which time no visible rings had formed. Portions of agar were transferred from the center and outer areas of the plates to tubes of broth. Growth in the tubes showed that the bacteria were viable in all portions of the plates. An additional 24-hour incubation gave definite rings in some plates. Transfers were made of agar blocks from the center and outer areas of plates showing ring formation. Growth occurred only in those tubes which received blocks from inside the rings. No growth was obtained from material outside the rings or from heavily seeded 48-hour plates showing no ring formation. This indicated that the bacteria in heavily seeded plates lost their ability to continue growth unless rings were formed.

In lightly seeded plates, those colonies separated a millimeter or two from each other often matured after 72 hours into typical white, convoluted colonies which failed to break down and likewise failed to grow in nutrient broth or on fresh agar slants. Frequently, however, individual colonies or groups of colonies broke down into the hyaline form from which growth was readily obtained on transfer to broth or agar.

Plates that contained fewer than 50 colonies and in which the colonies were several millimeters apart offered favorable conditions for long-continued growth. The cells in these colonies remained cultivable longer than 72 hours, but eventually they too matured and failed to grow when transferred to fresh culture media unless breakdown took place before sub-transfer.

Attempts to Induce Bacteria from Outside the Rings to Grow

Since microscopic examination of the organisms in colonies outside the rings suggested they might be viable but in a dormant state, preliminary attempts were made to rid the cells of their gelatinous matrix and test whether this might induce growth. Aliquots of nutrient glucose broth were adjusted to pH 7.6, 8.6, and 9.7. Colonies from outside typical rings were then placed in the broth tubes and incubated at 28°C. Examination of the tubes revealed that the capsular material surrounding the cells was broken down at pH 8.6 and 9.7; no growth, however, occurred in the tubes. Sub-transfers to fresh nutrient broth at the same pH as the original tube or to broth at pH 7.0 also gave no growth.

The bacterial cells were next treated by placing them in solutions containing a buffer salt at various concentrations. Tubes of 1 M K_2HPO_4 were diluted

with distilled water in such a way that each tube represented one-half the concentration of the preceding one. The first tube of the series contained 1 M K_2HPO_4 , while the tenth tube contained 0.002 M K_2HPO_4 . The buffer tubes were sterilized and later inoculated with material from outside the rings. Capsule dissolution took place at different rates depending on the concentration of K_2HPO_4 . The inoculum was sub-transferred from buffer to broth, care being taken to make the transfer as soon as the capsular material was dissolved. One ml. of material was transferred from each tube to a tube of fresh nutrient broth at pH 7.0. Material from the first tube was transferred after 2 minutes and material from the third tube after 15 minutes. The sixth tube of K_2HPO_4 represented a 0.031 M concentration and required an overnight treatment before the colonies were sufficiently broken down to warrant transfer to broth. No breakdown of the capsular material occurred at any greater dilution. Subsequent examination of the broth tubes revealed that no growth took place on sub-transfer, regardless of the treatment.

Mechanical separation of the cells from their confining matrix was next tried. Nutrient glucose broth in 50 ml. portions was placed in 125 ml. flasks containing a number of 5 mm. glass beads. The flasks were steam-sterilized at 15 pounds pressure for 20 minutes and then heavily inoculated with material from typical convoluted colonies which had not broken down. The flasks were shaken by hand until turbidity of the broth indicated that pulverization of the material had taken place. No growth occurred, however, in any of the flasks.

The possibility that a cold treatment might stimulate growth was considered. Petri plates containing 5-day-old colonies which had not broken down were placed in a refrigerator at 5°C. for 24 hours. The plates were then kept at 28°C. for 24 hours, and material from various portions of the plates was transferred to broth. No growth was obtained by this method.

A preliminary attempt was made to extract the alkaline material from Petri plates showing ring formation. It was thought that this material incorporated in broth might stimulate the cells and cause them to grow. Petri plates displaying well-developed rings were used. The agar within the rings was cut out of the plates, macerated, and placed in 20 ml. of nutrient broth for 30 minutes. The broth was then filtered through coarse filter paper to remove the agar and the filtrate passed through a Jena "5 auf 3" sintered glass filter. The filtrate was incubated 24 hours to insure sterility and then dispensed aseptically in 1 ml. portions to small sterile test tubes. These tubes were inoculated with material from plates showing no ring formation and incubated at 28°C. No growth resulted from this treatment.

Colonies from outside the rings were also inoculated into young bean plants, since the possibility existed that plant juice might be a more favorable medium than nutrient broth. No lesions developed, however, and it was concluded that the bacteria probably had not grown.

Since many of the above described treatments were of a preliminary nature, no definite conclusions were reached as to whether the bacteria outside the rings were living or dead.

DISCUSSION

The significance of ring formation and its relation to colony viability is not yet understood. The marked change in culture medium reaction within the rings, correlated with the change in consistency of the colonies, indicated that the organism had altered its metabolism in some manner. This may have been brought about by its ability to change the reaction of the medium within the rings to a pH level favorable for continued growth. The fact that colonies broke down first where the medium was thinnest supported this conclusion. The buffering system in thin areas of culture medium would be easier to change than in areas where the culture medium was thicker. Colonies outside the rings, where the medium was deep, would then become dormant or die because of inability to change the reaction of the medium during the active stage of growth.

On the other hand, the organism may act on the culture medium to form a toxic substance which kills or inhibits growth of the cells. The amount of this substance formed would be proportional to the density of bacterial growth and the quantity of medium available. The amount of toxic substance formed would then be greater where the agar was thickest. The quantity produced within the ring where the agar was thin would not be sufficiently great to cause death of the organism, particularly if the bacteria were capable of altering their metabolism and changing the culture medium reaction. The change in metabolism might permit the cells within the ring to continue growth.

Preliminary culture experiments for the purpose of determining the status of the bacteria outside the rings indicated that they might be dead. Microscopic examination, however, revealed that the cells appeared to be in better condition than those actively growing inside the rings.

This suggested a third possibility, that preparation for a long dormant period was adequate only in a deep medium and that under certain conditions this dormancy might be necessary for survival in nature.

SUMMARY

1. A gummy, convoluted colony type of *Phytomonas medicaginis* (Sackett) Bergey *et al.* var. *phaseolicola* Burkholder was observed to produce two different kinds of colonies in heavily seeded dilution plates. The two colony-forms were separated by rings, one form occurring inside and the other outside the rings.

2. The rings were usually irregularly shaped, varying in diameter from several centimeters to almost the width of the plate, and consisting of a narrow, slightly raised margin surrounding clear or partially clear central areas.

3. Ring formation was influenced by depth of medium, as was shown by imbedding slides or various-sized watch glasses in poured plates.

4. Rings formed in those portions of plates where the agar medium was thinnest. They rarely formed in plates containing a deep layer of agar.

5. A very striking change in reaction of the medium accompanied ring formation. The medium inside the rings gave an intensely alkaline reaction, while that outside the rings showed no change in reaction.

6. Studies on viability of the bacteria revealed that those inside the rings and from the rings themselves gave immediate growth when transferred to broth or agar. Bacteria outside the rings failed to grow even after prolonged incubation.

7. A number of experiments were tried to induce growth of bacteria outside the rings, but none of these met with success.

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PLATE I

(Photograph by J. A. Carlile)

FIG. 1. A convoluted colony type of *Phylomonas medicaginis* (Sackett) Bergey *et al.* var. *phaseolicola* Burkholder. $\times 1$.

FIGS. 2 TO 4. Typical rings formed in heavily seeded dilution plates.

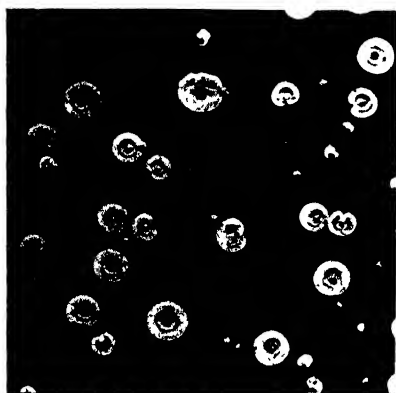


FIG. 1

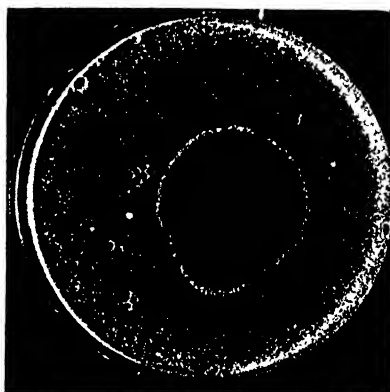


FIG. 2

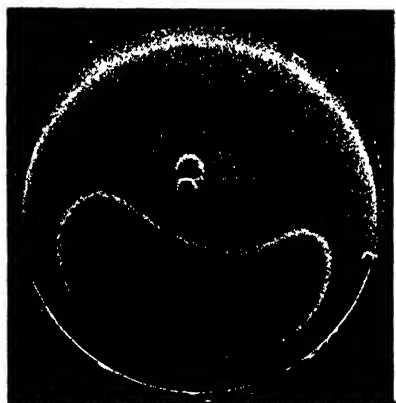


FIG. 3

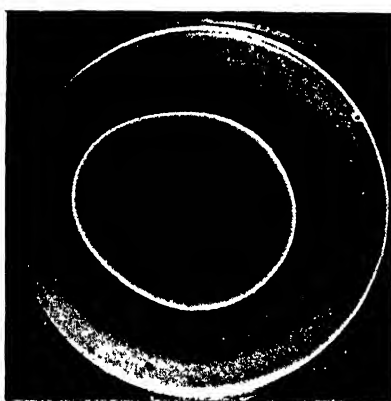


FIG. 4

(K. W. Kreitlow Heteromorphic Colonies and Ring Formation)

PLATE II

(Photograph by J. A. Carlile)

FIG. 5. Ring formed over a microscope slide imbedded in agar.

FIGS 6 TO 8. The distinct change in culture medium reaction as demonstrated by bromthymol blue paper laid on the agar surface. The ring forms a sharp line of demarcation between the inner part of the medium which is alkaline and the outer part of the medium which is acid.

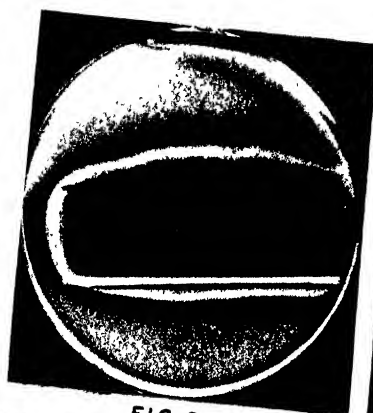


FIG. 5

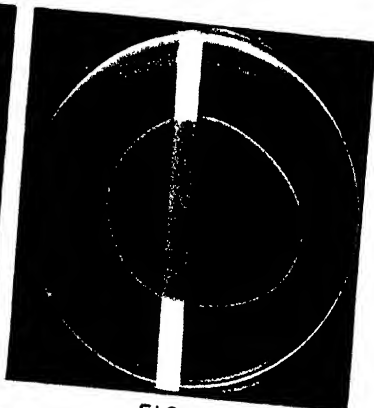


FIG. 6

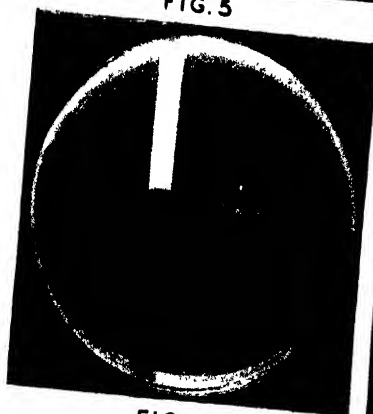


FIG. 7

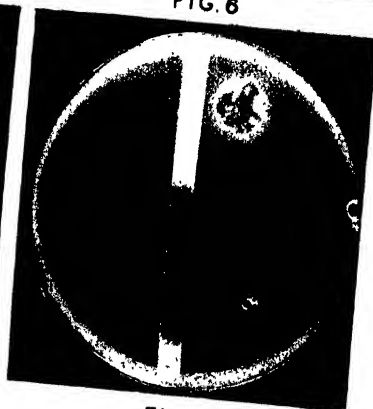


FIG. 8

(K. W. Kreitlow Heteromorphic Colonies and Ring Formation)

PASSAGE OF AIR THROUGH PLANTS AND ITS RELATION TO MEASUREMENT OF RESPIRATION AND ASSIMILATION*

By VIOLETTE F. C. GLASSTONE

*(From the Department of Animal and Plant Pathology of The Rockefeller Institute for
Medical Research, Princeton, New Jersey)*

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During the course of preliminary respiration experiments with entire plants, it was found that air passes readily through the tissues either upward or downward. Since this finding was of some importance in connection with work on respiration, the results obtained were thought to be of sufficient general interest to justify a report. Plants in earthenware porous pots filled with soil, the surface of which was covered with a low-melting-point hydrocarbon wax mixture, were used. Particular care was taken to see that the soil was completely covered and that a good seal was made around the stem. While the wax was still fluid, a glass respiration chamber was placed over the shoot, which was thus completely enclosed; the system was apparently airtight as soon as the wax set hard. Air from a compressed air main was first freed from carbon dioxide and then passed into the respiration chamber; it left via an exit tube which led to a carbon dioxide absorption tower and thence to a vacuum tap. The only other opening to the respiration chamber was for a manometer, to indicate the pressure; by manipulating the compressed air and the vacuum valves, a steady rate of flow at atmospheric pressure was maintained. In testing the apparatus for leaks, a porous pot containing soil but no plant was poured with wax as described and the chamber sealed into place. The apparatus proved to be perfectly airtight; and, in fact, the manometers were so very sensitive that special precautions had to be taken to minimize the inevitable changes in vacuum or air pressure.

When experiments were made with pots containing plants, negligible and zero amounts of carbon dioxide were taken up in the absorption towers; this result suggested that no respiration was taking place, a conclusion which was obviously incorrect. It was noticed, however, that the manometers attached to the chambers containing the plants had ceased to show the sensitive movements, since use of either vacuum or compression line produced only slight positive or negative pressures. Similar results were obtained by using plants

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from which the shoots had been excised, leaving only cut stumps protruding through the wax. These facts suggested that air was passing right through the plants.

Confirmation of the fact that air was able to pass readily through plants was obtained by replacing the porous pot with a non-porous glass container. After a few minutes, equilibrium was attained and the manometers became exceedingly sensitive. Under these conditions, normal respiration measurements could be made.

EXPERIMENTAL METHOD.—Experiments on the rate of passage of air through plants were carried out in the following manner. The specimen was placed in a porous pot with a respiration chamber sealed over the shoot as usual. A manometer containing *n*-butyl phthalate was fixed in position, and the only other opening was occupied by an exit tube leading first to a flow meter, next to a 20-liter glass jar which acted as a storage tank, and then to a T-tube connecting either to the vacuum tap or compressed air tap as desired. The vacuum tap was turned on until the manometer liquid just began to move; after a time the levels were noted and the suction increased. This procedure was continued until the maximum rate of flow measurable on the flow meter was reached. Analogous experiments were carried out using compressed air, instead of vacuum, and thereby reversing the direction of air flow. The levels in the manometer and flow meter at any rate of flow could be held stationary and the flow of air continued apparently indefinitely.

RESULTS.—Table 1 shows a typical set of readings obtained with a plant of *Nicotiana sylvestris* Speg. and Comes, of which the fresh shoot weighed 28.5 gm. and the area of under surface of leaves was about 878 sq. cm.

It will be observed from the figures in the last column that there was an approximate proportionality between the rates of air flow and the difference of pressure between the experimental chamber and the external atmosphere as measured by the manometer. The proportionality constant expressed in liters of air passing through the plant per hour per cm. of *n*-butyl phthalate is called the "rate coefficient" throughout this paper.

Similar results were obtained with all the plants tested: the data are summarized in table 2. The observations for each plant were reproducible over many hours; and where, as will be noted later, certain changes took place in the rate coefficient, these effects were also reproducible.

In some plants the rate coefficients were the same for either direction of flow, while in others there were differences in the two directions, *i.e.*, from leaf to root and root to leaf, respectively. Wherever two rate coefficients are given in column 4, as for example Oriental Poppy 5.5–35.0 liters per hour per cm. *n*-butyl phthalate, the air passed through the plant at the highest value for low rates of flow. When attempts were made to exceed a certain rate of flow, varying with each species, a resistance developed which diminished

the rate coefficient until a certain pressure was attained. After a time at this high pressure, a new steady state was established, and the air usually passed through the plant at the higher speed. In most of the plants which showed this effect, the change, at the high pressure, from slow to high speed was very sudden. The pressure at which the rapid change occurred was fairly constant for each species and could be repeated apparently indefinitely by allowing a period of time to elapse or by reversing the air current. For the purpose of determining whether the presence or absence of water within the plant or soil was a possible factor, a zinnia plant was well watered before being used.

TABLE 1
Passage of Air through Nicotiana sylvestris

	Rate of air flow (in liters per hour)	Difference in manometer levels (in cm. of butyl phthalate) ^a	Ratio of rate of air flow per cm. difference of pressure
Compressed air	7.5	0.5	15.0
	15.0	0.9	16.6
	22.5	1.5	15.0
	33.0	2.2	15.0
	46.5	2.9	16.0
	58.5	3.7	15.8
Vacuum	7.5	0.4	18.7
	15.0	1.0	15.0
	22.5	1.5	15.0
	33.0	2.2	15.0
	46.5	3.3	14.1
	58.5	4.4	13.3

^a One cm. of butyl phthalate is equivalent to about 10^{-3} atm. pressure.

The experiment was continued on the same plant for two days. No water was added during the period but, since it was left sealed in the chamber all the time, the gradual loss of water was not serious, and the plant remained in good condition to the end of the test. Quite early on the first day the rate coefficient for passage of air from root to leaf suddenly increased to the high value of 30 l./hr./cm. pressure; while the low value of 9 l./hr./cm. pressure continued for the passage of air in the opposite direction. It was demonstrated repeatedly that reversal of air flow, or the passage of time, allowed a resumption of the resistance to the flow of air from root to leaf, and that the sudden change in rate coefficient always occurred at the same pressures. It was not until the latter half of the second day, in the case of the flow in the direction from leaf to root, that the rate coefficient began to increase gradually until it approached that of the flow in the opposite direction, *i.e.*, 30 l./hr./cm. pressure.

In the case of *Nicotiana sylvestris* the leaves grow in a rosette, and the wax was poured so as to flow into the heart of the rosette and to cover all the leaf bases: no stem was above the surface of the wax. It was, therefore, certain

TABLE 2
Rate of Air Flow through Plants

Species of plant	Total shoot weight in gm.	Total leaf area in cm. ²	Rate coefficient expressed in liters per hour per cm. <i>n</i> -butyl phthalate		
			Actual	Per gram of fresh shoot weight	Per square cm. of leaf area
<i>Nicotiana tabacum</i> var. Samsun	52.5	1,150	150	2.9	0.130
	27.0	650	50	1.9	0.077
<i>N. sylvestris</i>	28.5	878	15.3	0.5	0.017
<i>N. glutinosa</i>	28	570	5.5	0.2	0.0096
Zinnia, Golden Gem:					
Vacuum	18.5	465	6.5-30 ^a	0.35-1.6	0.014 -0.065
Compressed air	18.5	465	9 -30 ^a	0.5 -1.6	0.019 -0.065
Poppy, Oriental	5.2	160	5.5-35 ^a	1.0 -6.7	0.034 -0.22
Aster, Shell Pink	14.5	639	7.5-13 ^a	0.5 -0.9	0.012 -0.02
Dianthus, China Pink	13.0	265	2 -20 ^a	0.15-1.5	0.0075-0.075
Crimson clover:					
Vacuum	24.0	760	85	3.5	0.11
Compressed air	24.0	760	55	2.3	0.07
Cabbage, Early Jersey Wakefield:					
Vacuum	52	1,002	6.5	0.125	0.0065
Compressed air	52	1,002	5-15 ^a	0.96-0.29	0.005-0.015
<i>N. rustica</i> :					
Vacuum	50.1	809	3.75	0.075	0.005
Compressed air	50.1	809	8	0.16	0.1
Cucumber, Henderson:					
Vacuum	18	447	10	0.55	0.022
Compressed air	18	447	1.5	0.08	0.0034
Tomato, Bonny Best:					
Vacuum	16	300	5	0.31	0.0167
Compressed air	16	300	1.5	0.09	0.005
Bean, Golden Cluster	13.6	394	4-12 ^a	0.3-0.9	0.01 -0.03
Wheat, Marquis Spring	6.5	253	4- 6.5 ^a	0.6-1.0	0.016-0.026

^a The explanation for the two rates is given in the text.

that the air must have passed through the leaves. Other experiments were devised to expose either stem, root, or leaf only above the wax surface.

Plants of sunflower, tomato, *Datura* and tobacco were grown until the leaves from the lower part of the stem had died back naturally; the upper portion of the stem with leaves was cut off and the cut surface sealed with wax. These plants were used with the stump varying from 18-25 cm. long protruding

above the wax. The rates of passage of air showed a proportionality as before, but the rate coefficients were considerably lower at 1 to 2 l./hr./cm. The stems were not able to allow a flow of air above an average of 10 l./hr. The tobacco stem, however, was exceptional, in that it would allow an air current of 60 l./hr. to pass from top to base with a rate coefficient of 8 l./hr./cm., if sufficient time was allowed for adjustment. The highest air flow in the reverse direction was 15 l./hr. with a coefficient of 3.5 to 4 l./hr./cm.

For roots only, tobacco plants which had been grown in nutrient solution were planted in sand, with some of the root above the surface; the stem and leaves were removed by cutting through the root region. Also the roots of dock plants, planted in soil, were exposed; the leaves and stem were cut away and the whole was waxed over, leaving the cut surface of the root uncovered. The rate coefficient for 3 cm. of exposed tobacco root was 30 l./hr./cm.; for the dock root with cut surface alone exposed it was 3 l./hr./cm.

For leaf only, the whole of a tobacco plant was waxed over except for one leaf of about 50 sq. cm. area. Even this small area allowed air to pass through in either direction at 3 l./hr./cm.

Although different plants exhibited certain differences of behavior, the experiments established the fact that the application of relatively small pressures will permit large volumes of air to pass through plant tissues of various kinds. The particular rate coefficient may, as with zinnia, be determined by the amount of water present in the plant or soil. The size of the plant is probably a determining factor (*cf.* the two tobacco plants), and so also is its age (*cf.* the seedlings of bean and wheat). It appears justifiable to conclude, however, that individual and specific differences in plants are largely differences of degree.

In order to test the airtightness of the wax seal around the stem and respiration chamber, tobacco plants were prepared as usual. Before the readings were observed, however, water was poured on to the top of the wax seal, making certain that the level of the water was above the level of the wax both on the stem and around the edge of the chamber. No bubbles of air came through the water, although the tests were repeated several times on different days and with different specimens. Several depths of water were tried on each occasion, and at all rates of flow from the lowest to the highest the readings were comparable to those already given, indicating clearly that no leak occurred in the wax seal, and that the air was passing through the plant.

DISCUSSION.—In these experiments no attempt was made to determine the actual path taken by the air through the plant. The experimental results are closely related to the facts demonstrated by Zimmerman *et al.* (1931) on the very rapid passage of gases, particularly ethylene, into and out of plants; to the ventilation of tree trunks, investigated by McDougal (1936); and also to the observation of Cannon (1925) in relation to roots and the aeration of soil.

The work described in this paper proves the ability of air to pass in either direction through the entire plant, and presumably through isolated or attached organs such as leaves. In the case of some plants, especially tobacco, very high rates of air passage were demonstrated with almost undetectable changes of pressure. This result was important in connection with the measurement of quantities of gases liberated or assimilated by plants.

SUMMARY

Seventeen species of plants were examined and found to permit the passage of air through their tissues both in the direction of leaf to root and root to leaf. There was an approximate proportionality between the amount of air passing and the applied pressure; and the results were reproducible. It was noted that age, size, and moisture condition of the plant were factors which appeared to affect the rate of passage. It is suggested that, while individual and specific differences occurred in ability to allow air to pass through tissues, those differences are believed to be matters of degree. The rapid passage of air through plants was demonstrated in connection with the measurement of carbon dioxide produced in respiration.

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THE HOMOGENEITY OF BUSHY STUNT VIRUS PROTEIN AS DETERMINED BY THE ULTRACENTRIFUGE*

By MAX A. LAUFFER

*(From the Department of Animal and Plant Pathology of The Rockefeller
Institute for Medical Research, Princeton, New Jersey)*

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Since the isolation of a protein possessing tobacco mosaic virus activity from diseased tobacco plants (6), viruses have been considered by some to be protein molecules. If a definition is accepted which requires that all of the molecules of a given substance be composed of the same kind and number of atoms arranged in the same way, one of the minimum conditions a virus protein preparation must satisfy in order to be considered molecular in nature is absolute homogeneity with respect to size and shape. As was early pointed out by Svedberg and his collaborators (9), the ultracentrifuge may be very useful for the determination of the degree of homogeneity of protein preparations.

When a protein solution is centrifuged under ideal conditions at high speed, the particles or molecules move towards the periphery of the centrifuge at a more or less uniform rate. The protein particles which were originally at the top of the column of solution form a boundary between protein solution and solvent, a boundary which sediments at the rate of the protein particles. If the centrifugation process is ideal, such boundaries become more and more diffuse as sedimentation proceeds. This is always due in part to the diffusion of the protein but may also be due in part to inhomogeneity. To demonstrate homogeneity by means of the ultracentrifuge, it is necessary to show that the observed boundary spreading can be accounted for entirely by the known diffusion rate of the material.

Eriksson-Quensel and Svedberg (2) were the first to study purified tobacco mosaic virus in the ultracentrifuge. They showed that the observed boundary spreading of one of the early samples prepared by chemical means by Stanley was entirely too great to be accounted for by diffusion on the assumption of any reasonable value for the rate of diffusion. Wyckoff (10) later studied the sedimentation of somewhat more favorable preparations of the virus isolated by differential centrifugation and con-

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cluded, by qualitative inspection of the sharpness of the sedimenting boundaries, that the virus was homogeneous. However, pictures of tobacco mosaic virus particles obtained more recently with the electron microscope almost always have shown that there is a distribution of particle lengths about a mean value (8). Although preparation of the mounts for the electron microscope may cause some breakage of the particles, it seems likely that qualitative inspection of the sharpness of a sedimenting boundary cannot be regarded as a satisfactory criterion of particle homogeneity.

Tomato bushy stunt virus protein, first isolated and crystallized by Bawden and Pirie (1), was early shown to consist of particles either spherical or nearly spherical in shape. Sedimentation equilibrium studies carried out by McFarlane and Kekwick (4) indicated that the distribution of the virus particles in a centrifugal field of about 150 *g* after a long period of centrifugation was approximately that which one would expect of a homogeneous material. This is a reasonable criterion of homogeneity but not a particularly sensitive one. Sedimentation velocity measurements at much higher centrifugal fields were also made by these workers, and it was observed at the qualitative level that the sedimenting boundaries were very sharp, again a reasonable though not critical indication of homogeneity.

In view of the facts (a) that no virus has heretofore been subjected to really critical physical tests for inhomogeneity without yielding evidence of inhomogeneity, (b) that bushy stunt virus is known not to possess a sufficient degree of inhomogeneity to be detected by less sensitive criteria, and (c) that the particles of this virus are approximately spherical, thereby making of it a favorable medium for a critical test, it was thought worth while to subject this material to the most exacting physical test for inhomogeneity possible with existing ultracentrifugation techniques. To that end, the spreading of a bushy stunt virus boundary in an ultracentrifugation experiment was measured very carefully and then was compared with the theoretical spreading calculated from the known diffusion constant of the virus on the assumption of absolute homogeneity.

EXPERIMENTAL

A sample of bushy stunt virus prepared by Dr. W. M. Stanley (7) was dissolved in 0.1 M phosphate buffer at pH 7 at a concentration of 3 mg. per ml. The solution was introduced into a 6 mm. sector-shaped centrifuge cell and spun in a Bauer and Pickels type air-driven ultracentrifuge (9) for 4 hours at 9000 R.P.M. The distance of the axis of rotation from the center of the cell was 65 mm. The temperature before and after the run was 20.9°. Photographs of the sedimenting boundary were taken at regular intervals according to the Lamm scale method (9). Various values of

scale distance were used. An exact duplicate run was made with the cell filled with buffer alone, and this served to define the base-lines for the scale diagrams.

Five of the scale exposures were chosen for analysis, and boundary diagrams of the conventional type were plotted out according to the general method outlined in Part III, B of Svedberg and Pedersen (9). The five comparable exposures on the buffer control run were used for determining the base-lines. The areas under the five curves were measured with a planimeter and were then reduced to a sort of standard state by correcting for scale distance and position of the center of the boundary with respect to the meniscus. The variation of these corrected areas about their mean was random, indicating that the scale-cell distance was fairly accurately known and also that there was no appreciable amount of material separating from the main component. The measured average area was 10 per cent greater than the value calculated from the refraction increment given by McFarlane and Kekwick (4), 0.00164, and the concentration of the virus, 3 mg. per cc., measured by the Kjeldahl method.

The theoretical boundary spreading was computed, in general, according to the method outlined in the section of Svedberg and Pedersen (9) just cited. The diffusion constant taken for the virus was that measured by Neurath and Cooper (5) by the Lamm method, 1.15×10^{-7} , corrected to water at 20°. It was assumed that diffusion began when the centrifuge attained its running speed of 9000 R.P.M. Since it took only 2 minutes for the acceleration of the machine and since the times of diffusion of the exposures studied varied from 50 to 220 minutes, no appreciable error could result from the possible incorrectness of this assumption. The curves were so computed that each one would have a corrected area equal to the average of the corrected areas measured for the actual boundary diagrams.

DISCUSSION

In Fig. 1 are presented the results of the investigation. As abscissas are plotted distances from the axis of rotation in cm. and, as ordinates, scale line displacements in μ . Each curve represents the position and degree of sharpness of the sedimenting boundary at a definite time after the beginning of sedimentation. The distance from the scale to the center of the cell, which is a measure of the magnification of the ordinate, varied from diagram to diagram. Both the times and the cell-scale distances for the various diagrams are indicated in the description of Fig. 1. The open circles are the experimental data describing the actual boundary spreading and the smooth curves represent the sharpness the boundaries ought to have if spreading were due solely to the diffusion of a strictly homogeneous material, calculated as outlined in the preceding paragraph.

The excellent agreement indicates that, contrary to an earlier opinion (3), the bushy stunt virus particles are indeed exceedingly homogeneous with respect to sedimentation rate and, therefore, with respect to size, shape, and density.

An attempt was made to evaluate somewhat more critically the meaning of the agreement here obtained between theoretical and observed boundary spreading. For the purpose of doing this, bushy stunt virus was visualized as being composed of a family of particles with a distribution of sedimentation rates obeying the normal frequency distribution law. Theoretical curves for the boundary after 220 minutes of sedimentation were then calculated for the cases in which the standard deviations of the distributions were 2 per cent and 5 per cent of the mean rate.¹ These theoretical boundary diagrams and the one computed on the assumption of absolute homogeneity are shown in Fig. 2. The actual boundary data, the same as those on the last curve of Fig. 1, are also presented. It may be seen that it is a little difficult to decide with certainty between the theoretical boundary diagrams calculated for absolute homogeneity and for a 2 per cent spread, but there can be no doubt that the one for a spread of 5 per cent is eliminated. Actually the data do favor the boundary computed for the case of absolute homogeneity. It can be concluded, then, that these experiments have shown that bushy stunt virus particles either are absolutely homogeneous with respect to sedimentation rate or are represented by a sedimentation rate distribution with a standard deviation no greater than 2 per cent of the mean. The physical meaning of this conclusion is clearer if this hypothetical variation in sedimentation rate is ascribed to a

¹ This problem was attacked in a non-rigorous manner, as follows: The sedimentation and diffusion processes were visualized as taking place independently, in sequence. First, the boundary was pictured as moving from the meniscus to its final position in practically no time. After this the boundary should be represented approximately by a normal curve with a standard deviation equal to σ/S times the distance the boundary moved, where S and σ are the mean and the standard deviation of the sedimentation rates. Since the standard deviation of a normal curve is equal to $\sqrt{2Dt}$, where t is time and D is the diffusion coefficient of the material whose boundary is represented by that normal curve, one can calculate a time, t_0 , which it would have taken for the boundary to acquire its current state of sharpness by a process of diffusion. The diffusion process was next visualized as taking place for a time t equal to that of the experiment, while the boundary remained stationary. The final boundary diagram should have approximately the shape it would have had if diffusion had proceeded for a time $t + t_0$. In this process the same corrections were applied to D that are used in obtaining the theoretical diagram for the case of a strictly homogeneous material. To make the calculations as simple as possible, it was assumed that a small variability in the sedimentation constant will not cause a measurable change in the diffusion rate, an assumption justified by the relative insensitivity of the diffusion process to small changes in particle size and shape.

variation in the radius of the particles. A 2 per cent variation in sedimentation rate would correspond to a 2 per cent variation in the square of the radius or to about a 1 per cent variation in the radius and in the diameter. Hence, these data may be reinterpreted somewhat more loosely to mean that bushy stunt virus particles either are of identical size or are repre-

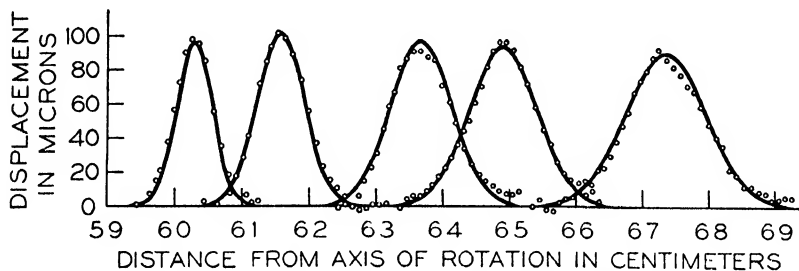


FIG. 1. Theoretical *versus* actual boundary spreading in sedimentation experiment on bushy stunt virus. The circles are experimental points obtained by the Lamm scale method. The smooth curves are theoretical boundary diagrams calculated from the known diffusion constant. The times and the scale-cell distances for the successive boundary curves, beginning at the left, are 50 minutes, 2.1 cm.; 85 minutes, 3.1 cm.; 135 minutes, 4.1 cm.; 150 minutes, 4.6 cm.; 220 minutes, 5.6 cm.

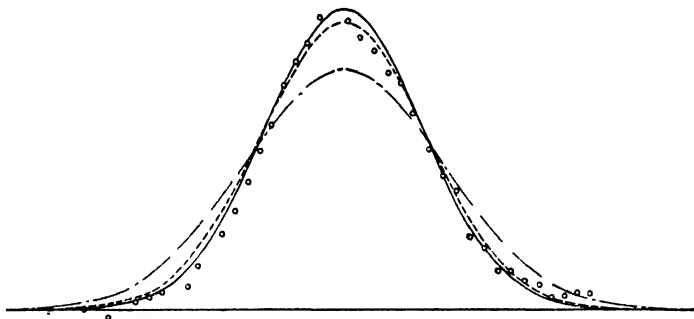


FIG. 2. Theoretical boundary curves computed on the assumption that the sedimentation rate of the bushy stunt virus particles may be represented by normal curves with standard deviations of 0 per cent (solid line), 2 per cent (dash line), and 5 per cent (dot and dash line) of the mean sedimentation rate, compared to the actual boundary diagram obtained after 220 minutes of centrifugation (circles).

sented by a distribution of diameters with a standard deviation no greater than 1 per cent of the mean diameter. It is unlikely that very many of the proteins generally conceded to be in a molecular state of dispersion have been demonstrated to be homogeneous within limits any narrower than this. Therefore, the case for believing that bushy stunt virus protein particles are molecules is as good as for any protein.

SUMMARY

The spreading of a tomato bushy stunt virus boundary during a sedimentation velocity experiment was measured by the Lamm scale method and was compared to the theoretical spreading one should expect due to the known diffusion rate of the material if the virus is strictly homogeneous. The results showed that the boundary spreading can be accounted for satisfactorily in terms of diffusion. A more detailed consideration of the data indicated that the method as here applied was sufficiently sensitive to exclude the possibility of the virus particles being represented by a normal size distribution function with a standard deviation of the particle diameters greater than 1 per cent of the mean diameter. In view of this result, it was concluded that the justification for believing that bushy stunt virus protein particles are strictly homogeneous with respect to size, shape, and density and that they, therefore, may be molecules is as good as for any protein.

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A SENSITIVE CHECK VALVE

By MAX A. LAUFFER

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

Before carrying out electrophoresis or diffusion experiments by methods involving the study of a boundary between solvent and solution, it is necessary to bring a small volume of the colloidal solution under consideration into ionic equilibrium with the solvent, usually a dilute buffered salt solution. Although this can be accomplished by simple stationary dialysis if sufficient time is allowed, it was thought likely that the equilibrium could be attained much more rapidly if some such device as the rocking dialyzer of Kunitz and Simms¹ were used. All that was required was some means of causing the buffer solution to circulate constantly past the dialyzing membrane. As was first pointed out to the author by Dr. D. A. MacInnes, the necessary energy for such circulation can be derived from the rocking motion of the dialyzer. A well-built rocker with a reliable source of power,² a small reservoir attached to one end of the table of the rocker, a larger reservoir to hold the bulk of the liquid, some rubber tubing and two check valves to render flow unidirectional constitute all the essential features of the set-up used in our laboratory to obtain the desired circulation. A diagrammatic representation of the assembly is shown in Fig. 1. In order to make the apparatus work, the level of the liquid in the larger reservoir must be intermediate between the upper and lower positions of the smaller reservoir and the check valves must be sufficiently sensitive to be opened and closed by small pressure gradients. A very simple and highly sensitive check valve suitable for such purposes was designed and constructed with the aid of Mr. William Duthie, machinist in our laboratory. Because of the satisfactory service the check valve has given, it was thought desirable to publish a description of it.

The valve consists of nothing more than a very thin sheet of rubber resting against the opening of a hole drilled into a "lucite" rod. A slight pressure tending to cause a liquid to flow out of the hole pushes the rubber film away, but an equally small pressure in the opposite direction causes it to cover the opening securely, preventing the flow of liquid in that direction. As is illustrated in Fig. 2, two modifications of the valve were constructed. The simpler of the two, No. 1, was cut from a 2-inch section of a 3/4-inch "lucite" rod. After drilling a 3/16-inch hole (a) through the center of the rod from one end to within 3/16 inch of the opposite end, hereafter designated as the head, a 1/8-

¹ M. Kunitz and H. S. Simms, *Jour. Gen. Physiol.*, 11: 641, 1928.

² A 1/80 HP universal motor with a 1:595 reduction gear manufactured by Bodine Electric Company of Chicago may be used for this purpose.

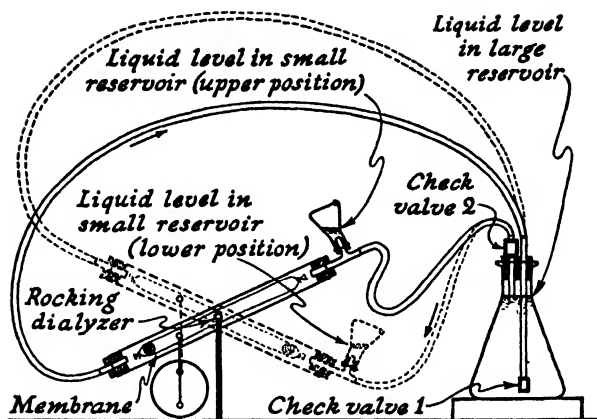


FIG. 1. Diagrammatic representation of assembly for rocking circulating dialysis.

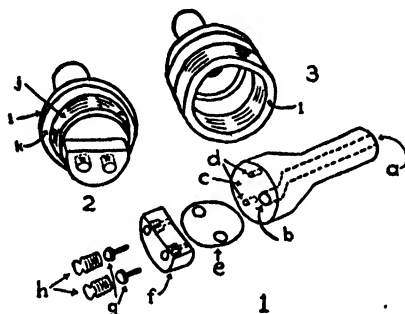


FIG. 2. The check valves. 1. Valve No. 1 shown unassembled. 2. Valve No. 2 shown assembled. 3. Cap for valve No. 2.

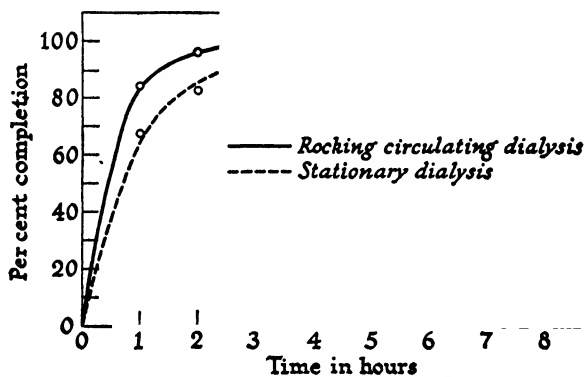


FIG. 3. Comparison of the rates of equilibration by rocking circulating dialysis and by stationary dialysis.

inch hole (b) was drilled diagonally from near the edge of the head end so that it connected with (a). The rod was then turned down to the shape indicated in the figure, the head retaining its 3/4-inch diameter and the small end being reduced to about 7/16 inch. The surface (c) was polished thoroughly. Two holes (d) were then drilled into the head as illustrated and tapped to accommodate No. 1-72 screws. A rubber diaphragm (e), about .01 inch thick, was cut to fit the surface (c), and holes were punched in it to correspond to those at (d) in the head. This diaphragm was fastened onto the head of the valve with "lucite" block (f), cut from a 3/4-inch rod and held in place with two No. 1-72 brass screws (g). The holes in the block accommodating the screws were countersunk and threaded to take the "lucite" plugs (h). Valve No. 2, which is slightly more complicated than No. 1, is shown assembled. The rubber flap is shown resting against the opening of hole (b). A slight pressure from within will bend the diaphragm away, but a slight pressure in the opposite direction will cause the flap to cover the opening securely. Valve No. 1 can be used only on the end of a tube conducting liquid into some sort of reservoir. Valve 2, which is identical in principle, was cut from a 1 1/8-inch "lucite" rod and was constructed in such a manner that a cap, 3, could be fitted over it, permitting it to be introduced at any point in a circulation system. A collar (i) was left at the base, and the shoulder (j) was threaded. The cap, 3, which screws onto the valve, 2, was cut from a similar rod. By polishing the surface of the collar (k) and the edge of the cap (l), it was possible to obtain a perfectly tight seal without the use of gaskets, if a little vaseline was applied to the threads before assembling.

Because of the elasticity of the rubber flap, the valve can be used in any position. Pressure differences as low as half a centimeter of water have been found to be sufficient for its operation. Constructed as it is so that only rubber and lucite are exposed, it can be used with most aqueous solutions, excepting concentrated acids and bases, and with those organic liquids which attack neither "lucite" nor rubber. Suitable materials could probably be found for making a similar valve for almost any special purpose.

The advantage of rocking circulating dialysis as here described over stationary dialysis for the equilibration of electrolyte concentrations was demonstrated by dialyzing 15 cc portions of distilled water against 2 liters of 0.2 M NaCl solution for various times using (a) rocking circulating dialysis and (b) stationary dialysis. The latter was accomplished by simply suspending the dialyzing bag near the bottom of the salt solution and allowing it to stand unagitated. Electrolyte concentrations after dialysis were estimated by measuring conductivities. As may be seen in Fig. 3, practical equilibrium is reached in about 3 hours by the rocking method but only in something more than 8 hours by the stationary method. In equilibrating viscous materials like protein solutions with electrolyte solutions, the advantages of the rocking method proved to be even more pronounced.

AN ALIGNMENT CHART FOR THE COMPUTATION OF ULTRACENTRIFUGATION RESULTS

By HOWARD K. SCHACHMAN*

(From the Department of Animal and Plant Pathology of The Rockefeller
Institute for Medical Research, Princeton, New Jersey)

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The discovery made by Svedberg (1) that the ultracentrifuge could be adapted to the study of proteins has resulted in wide-spread use of this instrument. A convenient way of describing sedimentation data is in terms of the sedimentation constant, S , originated by Svedberg. This quantity is simply the sedimentation velocity the material would have in a unit centrifugal field.

$$S = \frac{dx}{dt} \cdot \frac{1}{\omega^2 x} \quad (1)$$

Since the sedimentation rate of a given material is a function of the density of the solution and the solute and of the viscosity of the solvent, as well as of the magnitude of the centrifugal field, it is necessary to express sedimentation constants in terms of a reference solvent, such as water at 20°.

$$S_{20}^0 = \frac{dx}{dt} \cdot \frac{1}{\omega^2 x} \cdot \frac{\eta_t}{\eta_{20}^0} \cdot \frac{1 - V_{20} \rho_{20}^0}{1 - V_t \rho_t} \quad (2)$$

The meanings of the various terms in these two equations are defined by Svedberg (1). When Equation 2 is integrated, S_{20}^0 is expressed as a function of $\log_e x$, t , and the other variables listed above. It is obvious that considerable arithmetic is involved in the evaluation of sedimentation constants in this manner.

It has been the experience of the author that laborious routine computations of this sort can often be reduced to very simple operations by using some sort of an alignment chart (2). If one constructs three parallel equidistant lines perpendicular to and intersecting a base-line, then, by simple geometry, any line connecting points on the outside lines will intersect the center line at a point whose distance above the base-line is one-half the sum of the distances of the other two points above the base-line. If equal scales are laid out on the two outside lines, A and B , and a scale of half the magnitude is laid out on the inside line, C , then the numerical value

* Technical assistant in the Laboratory of Plant Pathology.

of C is equal to the sum of the numerical values of A and B (3). A device of this sort, commonly referred to as a nomograph or an alignment chart, is unusually well adapted to performing mathematical operations such as evaluating u when $u = x + y - z$. Since the sum of $x + y$ is the only quantity represented on the center scale and since its value is not recorded in a case such as this, the scale on the center line can be omitted. Similarly, an alignment chart can be constructed to solve the equation $Au = Bx^lCy^n/Dz^m$, where A, B, C, D, l, m , and n are constants. In this example, the values of each variable would be plotted on a logarithmic scale, expanded by the value of its particular exponent, and displaced vertically by the value of the logarithm of its coefficient.

Equation 1 may be integrated approximately as follows (1): $S = 1/\omega^2 \bar{x} \cdot \Delta x/\Delta t$, where Δx and Δt are small but finite increments of x and t , respectively, and \bar{x} is the average value of x in the interval between x and $x + \Delta x$. It can be shown that, if the experimental conditions are so chosen that Δx does not exceed $\bar{x}/10$, the error attending this approximation is less than 0.1 per cent. By inverting and by converting angular velocity to R.P.M., velocity to mm. per minute, and S to units 10^{13} times the fundamental unit,¹ this equation becomes

$$\frac{1}{S} = \frac{\pi^2}{1.5 \times 10^{13}} \frac{n^2 \bar{x}}{\Delta x/\Delta t}$$

where n represents R.P.M. This equation can be written in the form,

$$\frac{A}{S} = \frac{Bn^2 C \bar{x}}{D(\Delta x/\Delta t)}$$

where A, B, C , and D are arbitrarily assigned constants obeying the sole restriction, $BC/AD = \pi^2/(1.5 \times 10^{13})$.

$$\therefore [\log A - \log S] = [\log B + 2 \log n] + [\log C + \log \bar{x}] - \left[\log D + \log \frac{\Delta x}{\Delta t} \right]$$

Since Equation 1 could be expressed in the above form, an alignment chart for its solution could be constructed, as is shown in the right half of Fig. 1. On Column I, $[\log D + \log \Delta x/\Delta t]$ is plotted as a function of the variable, $\Delta x/\Delta t$, and $[\log B + 2 \log n]$ as a function of n or R.P.M. On Column III, $[\log A - \log S]$ is plotted as a function of S and $[\log C + \log \bar{x}]$ as a function of \bar{x} . Convenient values, subject to the restriction stated above, were chosen for the constants A, B, C , and D .

¹ By unanimous vote at the conference on "The ultracentrifuge" held by the New York Academy of Sciences on November 14-15, 1941, it was agreed that it be proposed that this unit, 10^{13} times the fundamental unit, be called the Svedberg. The alignment chart here described gives the results in Svedbergs.

With this portion of the alignment chart, sedimentation constants can be computed from ultracentrifuge data. If the sedimentation constants are to be useful for further interpretation, however, they must be corrected for the viscosity and the density of the solvent, as shown by Equation 2. This correction can be carried out in two steps,

$$S_\eta = S \cdot \frac{\eta_t}{\eta_{20}^0} \quad (3) \quad \text{and} \quad S_{20}^0 = S_\eta \cdot \frac{1 - V_{20}\rho_{20}^0}{1 - V_t\rho_t} \quad (4)$$

where S_η is the sedimentation constant corrected only for viscosity. One may assume that $\eta_t = [\eta/\eta_0]_{T_t} \times \eta_0$, where $[\eta/\eta_0]_{T_t}$ is the ratio of the viscosity of the solvent to that of water at some convenient temperature, T_t , and η_0 is the viscosity of water at the temperature of the solvent at the time of the experiment. Hence, Equation 3 may be written,

$$S_\eta = S[\eta/\eta_0]_{T_t} \times (\eta_0 = f(T))/(\eta_{20}^0 = 0.010087)$$

$$\therefore [\log E - \log S_\eta] = [\log A - \log S]$$

$$+ [\log F - \log \eta_0 = f(T)] - [\log G + \log [\eta/\eta_0]_{T_t}]$$

where A , E , F , and G are arbitrarily assigned constants obeying the sole condition that $AF/EG = \eta_{20}^0 = 0.010087$. In this form an alignment chart was constructed for the solution of Equation 3 as shown on Columns III and V. Here the bracketed quantities in the above equation are plotted as functions of the respective variables S_η , S , T , and $[\eta/\eta_0]_{T_t}$ (η/η_0 in the figure). The relationship between η_0 and T that was used was that given by Svedberg and Pedersen (1).

Equation 4 yet remains to be solved. It can be written in the form $[\log H - \log S_{20}^0] = [\log E - \log S_\eta] + [\log J - \log (1 - V_{20}\rho_{20}^0)] - [\log K - \log (1 - V_t\rho_t)]$, where $EJ/HK = 1$. Columns V and VII of Fig. 1 are the alignment chart for the solution of this equation. The bracketed quantities in the above equation are plotted as functions of the respective variables, S_{20}^0 , S_η , V_{20} , and $V_t\rho_t$.

Since $\log V_t\rho_t = \log V_t + \log \rho_t$ and $[\log L + \log V_t\rho_t] = [\log M + \log V_t] + [\log N + \log \rho_t]$, where $MN/L = 1$, $V_t\rho_t$ can be evaluated from V_t and ρ_t by constructing three equidistant parallel lines, with equal logarithmic scales representing V_t and ρ_t , respectively, on the extremities, and a logarithmic scale of half the dimensions representing $V_t\rho_t$ on the central column. Columns VIII, IX, and X represent the alignment chart for the valuation of $V_t\rho_t$. The lines between Columns IX and V connect values of $V_t\rho_t$ on Column IX with the corresponding values of $\log K - \log (1 - V_t\rho_t)$ on Column V.

To facilitate the use of this alignment chart, a photostatic copy was made and was mounted on a bakelite board.² Narrow slits were cut

² Photostatic copies of Fig. 1, 12 × 18 inches in size, can be obtained from the author.

through the photostat and into the bakelite in the positions of the three vertical lines, Columns II, IV, and VI, of Fig. 1. A brass key was cut to fit the channels and slide through them freely. A long thin strip of celluloid with a black hair-line through its center parallel to its two long sides was then fastened at its exact center to the brass key by means of a pivot. This device makes it possible to use the alignment chart without marking it in any way. Fig. 2 is a photograph of the mounted alignment chart showing the hair-line assembly.

The use of the alignment chart for the calculation of sedimentation constants may be illustrated with the following example. A sedimenta-

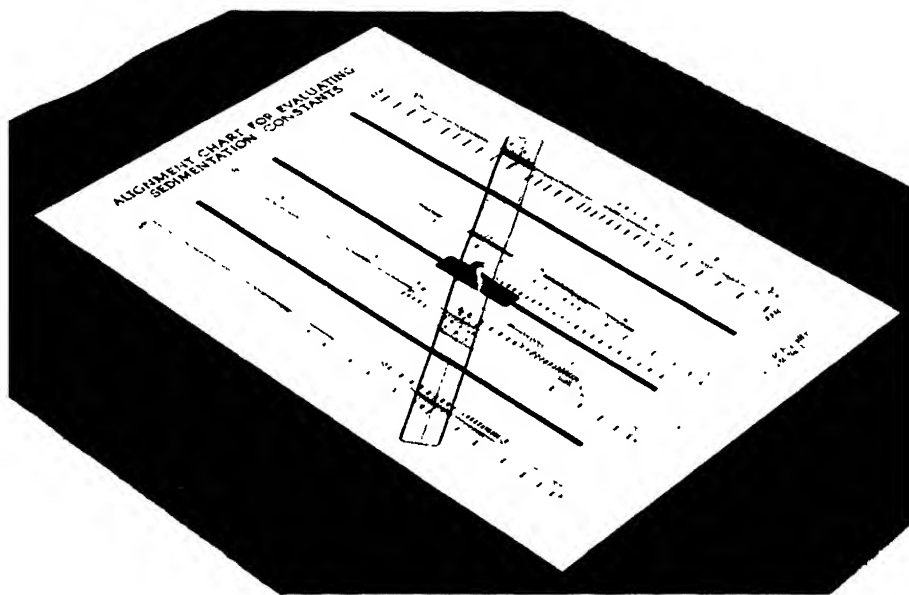


FIG. 2. Photograph showing the alignment chart mounted on a bakelite panel and equipped with convenient hair-line assembly.

tion experiment was carried out with tomato bushy stunt virus dissolved in 0.2 M NaCl and 0.01 M phosphate buffer at pH 7. The temperature throughout the run was $22.8^\circ \pm 0.2^\circ$. The centrifuge was operated at a constant speed of 18,500 R.P.M. The density of the solution was 1.01, the relative viscosity of the solvent, $[\eta/\eta_0]_r$, was 1.02, and the partial specific volume of the virus at 20.0° and at 22.8° was about 0.739. The boundary was photographed at 10 minute intervals. Other pertinent data are presented in Table I.

From the data recorded in the first two columns of Table I, \bar{x} and $\Delta x/\Delta t$ can readily be evaluated. For each value of \bar{x} and $\Delta x/\Delta t$, S was evaluated

by means of the alignment chart. This was accomplished by centering the hair-line assembly on Column II, by moving it into position so that 18,500 on the R.P.M. scale in Column I can be lined up with the particular value of \bar{x} , say 5.762 cm., on Column III. Then the hair-line was rotated about its pivot until the line coincided with the proper value of $\Delta x/\Delta t$ on Column I, in this case 0.175 mm. per minute. When this was done, the hair-line also crossed the sought value of S on Column III, 135 in this example. This process was repeated for all of the pairs of \bar{x} and $\Delta x/\Delta t$ recorded in Table I, and the resulting values of S were averaged. In the last two columns of Table I, the values of S determined with the chart and those calculated arithmetically are compared. The agreement is seen to

TABLE I
Typical Sedimentation Data

t	x	\bar{x}	$\frac{\Delta x}{\Delta t}$	S , chart	S , calculated
<i>min.</i>	<i>cm.</i>	<i>cm</i>	<i>mm. per min.</i>		
0	5.675	5.762	0.175	135	135
10	5.850	5.943	0.187	140	140
20	6.037	6.134	0.195	142	141
30	6.232	6.328	0.193	135	135.5
40	6.425	6.522	0.194	131.5	132
50	6.619				
Average				136.7	136.7

be excellent. The average value of S was next corrected for viscosity. This was done by centering the hair-line assembly on Column IV. The average value of S , 136.7, on Column III was aligned with 22.8 on the T scale on Column V, the hair-line was rotated and connected with 1.02 on the $[\eta/\eta_0]_{T_1}$ (η/η_0 on the figure) scale on Column III. The line was found to intersect Column V midway between 0.70 and 0.71 on the $V_{\rho t}$ scale. This point was recorded. If the S_{η} scale were continuous in this region, we could have read off the value of S_{η} directly. However, since the numerical value of this function is of no use, it is satisfactory to let it be represented by the point midway between 0.70 and 0.71 on the V_t scale. Next, it was necessary to evaluate $V_{\rho t}$. This was done by centering the

hair-line assembly in either Column IV or VI, and then by aligning the value 1.01 on the ρ_i scale on Column X with 0.739 on the V_i scale on Column VIII. The value of V_{ρ_i} was found at the point of intersection of the hair-line with the V_i scale on Column IX. This value, in this case 0.746, was also recorded. In order to carry out the final evaluation of S_{20}^0 , the hair-line assembly was then centered on Column VI, and the value of S_{ρ_i} , represented by the point midway between 0.70 and 0.71 on the V_{ρ_i} scale of Column V, was aligned with the value of V_{20} on Column VII, 0.739. The hair-line was rotated and connected with the value of V_{ρ_i} on Column V, 0.746, and the value of S_{20}^0 was then read off at the intersection of the hair-line with Column VII. The value in this case was 134. When S was corrected arithmetically, the value of S_{20}^0 was found to be 134. In another sample computation in which values near the ends of the scales were used, the value computed by means of the alignment chart was found to agree within about 1 per cent with the true value computed arithmetically.

As was demonstrated in the examples just discussed, with the aid of the alignment chart it was possible to compute the corrected sedimentation constant of bushy stunt virus without referring to anything except the original data. All of the auxiliary information needed is incorporated into the device itself. It was even possible to perform some necessary operations which cannot be carried out with other devices such as the standard slide-rule—for example, the evaluation of the expression $(1 - V\rho)$ from V and ρ . When these and other factors are taken into consideration, it is obvious that the calculation of sedimentation constants from experimental data by means of the alignment chart is a much simpler and faster process, on the whole, less subject to mistakes, than any of the more direct arithmetic processes, no matter how well systematized. The actual time required to perform the operations described above is about 3 minutes. If the computation is well organized and various tables of data are before the operator, the same operations can be carried out in about 10 minutes with the slide-rule. If the exact integral of Equation 1 is used, it is necessary to convert distances into logarithms and the time of computation is thereby considerably increased. On the whole, therefore, the alignment chart is capable of saving considerable time, and this, as has been demonstrated, can be realized without any appreciable sacrifice of precision.

The author wishes to express his appreciation to Dr. Max A. Lauffer for encouragement during the course of this work and for aid in the preparation of the manuscript.

SUMMARY

An alignment chart for the evaluation of sedimentation constants from sedimentation data was constructed and its use in a sample computation was described. It was demonstrated that this device is accurate, convenient, and capable of effecting a considerable economy in time.

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